THE CHEMISTRY OF ENZYME ACTIONS

SECOND AND REVISED EDITION

BY

K. GEORGE FALK

HARRIMAN RESEARCH LABORATORY
The Roosevelt Hospital
NEW YORK

American Chemical Society
Monograph Series

BOOK DEPARTMENT

The CHEMICAL CATALOG COMPANY, *Inc.*
19 EAST 24TH STREET, NEW YORK, U. S. A.
1924

Printed in the United States of America by
J. J. LITTLE AND IVES COMPANY, NEW YORK

GENERAL INTRODUCTION

American Chemical Society Series of Scientific and Technologic Monographs

By arrangement with the Interallied Conference of Pure and Applied Chemistry, which met in London and Brussels in July, 1919, the American Chemical Society was to undertake the production and publication of Scientific and Technologic Monographs on chemical subjects. At the same time it was agreed that the National Research Council, in coöperation with the American Chemical Society and the American Physical Society, should undertake the production and publication of Critical Tables of Chemical and Physical Constants. The American Chemical Society and the National Research Council mutually agreed to care for these two fields of chemical development. The American Chemical Society named as Trustees, to make the necessary arrangements for the publication of the monographs, Charles L. Parsons, Secretary of the American Chemical Society, Washington, D. C.; John E. Teeple, Treasurer of the American Chemical Society, New York City; and Professor Gellert Alleman of Swarthmore College. The Trustees have arranged for the publication of the American Chemical Society series of (a) Scientific and (b) Technologic Monographs by the Chemical Catalog Company of New York City.

The Council, acting through the Committee on National Policy of the American Chemical Society, appointed the editors, named at the close of this introduction, to have charge of securing authors, and of considering critically the manuscripts prepared. The editors of each series will endeavor to select topics which are of current interest and authors who are recognized as authorities in their respective fields. The list of monographs thus far secured appears in the publisher's own announcement elsewhere in this volume.

The development of knowledge in all branches of science, and especially in chemistry, has been so rapid during the last fifty years and the fields covered by this development have been so varied that it is difficult for any individual to keep in touch with the progress in branches of science outside his own specialty. In spite of the facilities for the examination of the literature given by Chemical Abstracts and such compendia as Beilstein's Handbuch der Organischen Chemie, Richter's Lexikon, Ostwald's Lehrbuch der Allgemeinen Chemie, Abegg's and Gmelin-Kraut's Handbuch der Anorganischen Chemie and the English and French Dictionaries of Chemistry, it often takes a great deal of time to coördinate the knowledge available upon a single topic. Consequently when men who have spent years in the study of important subjects are willing to coördinate their knowledge and present it in concise, readable form, they perform a service of the highest value to their fellow chemists.

It was with a clear recognition of the usefulness of reviews of this character that a Committee of the American Chemical Society recommended the publication of the two series of monographs under the auspices of the Society.

Two rather distinct purposes are to be served by these monographs. The first purpose, whose fulfilment will probably render to chemists in general the most important service, is to present the knowledge available upon the chosen topic in a readable form, intelligible to those whose activities may be along a wholly different line. Many chemists fail to realize how closely their investigations may be connected with other work which on the surface appears far afield from their own. These monographs will enable such men to form closer contact with the work of chemists in other lines of research. The second purpose is to promote research in the branch of science covered by the monograph, by furnishing a well digested survey of the progress already made in that field and by pointing out directions in which investigation needs to be extended. To facilitate the attainment of this purpose, it is intended to include extended references to the literature, which will enable anyone interested to follow up the subject in more detail. If the literature is so voluminous that a complete bibliography is impracticable, a critical selection will be made of those papers which are most important.

The publication of these books marks a distinct departure in the policy of the American Chemical Society inasmuch as it is a serious attempt to found an American chemical literature without primary regard to commercial considerations. The success of the venture will depend in large part upon the measure of coöperation which can be secured in the preparation of books dealing adequately with topics of general interest; it is earnestly hoped, therefore, that every member of the various organizations in the chemical and allied industries will recognize the importance of the enterprise and take sufficient interest to justify it.

AMERICAN CHEMICAL SOCIETY

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PREFACE TO SECOND EDITION

The new edition of this Monograph was prepared at the request of the Publisher and of the Editors of the Series of Scientific Monographs. Work is in active progress in the field of Enzyme Actions in a number of laboratories in Europe, in Japan, in this country, and elsewhere. The available material is increasing rapidly. Nevertheless, the subject has not attained, or even approached, a standardized, much less static, condition, for purposes of presentation.

The point of view from which this edition has been prepared is essentially the same as that of the former edition. The view which is emphasized particularly is the interdependence of various lines of chemical study. Enzyme actions may be treated as a group of chemical reactions analogous to other chemical changes. The substances taking part in such actions are an integral part of chemical science. Such considerations justify the inclusion of a treatise on Enzyme Actions in a series of Monographs of Chemistry. Although the subject matter cannot be presented in as well-rounded and final (?) a form as many might desire, for others, this very incompleteness together with the fact that the topic is a living one, subject to development, growth, and change, will add interest to its future study.

The study of enzyme actions is of importance not only in connection with, and as a part of, chemical science, but also in its biological aspects because of the bearing of such actions upon the chemical changes occurring in life processes. The development of the knowledge of the chemical phenomena underlying living matter is bound up intimately with a knowledge of enzyme actions. A number of results bearing on this question are presented in Chapter IX and may serve to indicate the possibilities of such studies.

Considerable new material has been incorporated in this edition, at the same time the essential parts of the former edition have been retained. Chapter IX is entirely new. The more recent studies of a number of investigators in this field have been included as far as practicable. While it has been impossible to include the work of

all, or even of the majority, it is hoped that the references which are given are sufficiently numerous and complete to enable others who may be interested to enter upon and to pursue studies along these lines.

The author wishes again to thank those who were mentioned in the Preface of the former edition for their aid and encouragement. At the same time, a special word of thanks must be extended to the following, who gave freely of their time, offering advice relative to the various questions which presented themselves:—Miss Grace McGuire and Miss Helen Miller Noyes of the Harriman Research Laboratory; Professors George B. Pegram and John M. Nelson of Columbia University; and Dr. John H. Northrop of the Rockefeller Institute for Medical Research.

The author also wishes to express his appreciation to the Editor of the Series of Scientific Monographs, as well as to his Associates on the Editorial Board, and to the Publisher, for the uniform courtesy and kindness which they have shown, and for the advice and suggestions offered by them in connection with this monograph.

A number of figures reproduced in Chapter IX have been published in papers appearing in the Journal of Biological Chemistry. Thanks are due the Journal for the use of the plates of these figures.

July 15, 1924.

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THE CHEMISTRY OF ENZYME ACTIONS

I.—Introduction

Enzymes may be defined as catalysts found in living matter. The study of the chemical nature of substances which occur in animal and vegetable matter and the changes these substances undergo during life processes, brought about the view that agents are present in living organisms which are capable of changing the velocities of certain definite chemical transformations in the material which is present. Without entering into the historical development of the discovery of various enzyme actions, or the relation between enzymes and ferments, it may be stated that the view is generally accepted that enzymes are catalysts; that they are found in, and therefore produced by, living organisms; and that they are not living in that they do not in themselves possess the powers of growth and reproduction in the ordinary meanings of these terms.

Enzymes and enzyme actions may be studied from various points of view. In the first place, attention may be fixed upon a certain chemical reaction and a search made for animal and vegetable materials from different sources such as tissue extracts, vegetable extracts, etc., which increase the velocity of the chemical change. Secondly, a definite preparation may be tested with a variety of chemical reactions in order to determine the different enzyme actions it possesses. These methods of investigation have been carried on very extensively and have yielded a rich harvest. Innumerable reactions may be listed whose velocities are modified by the addition of one or another of the enzyme preparations. This descriptive method of study must necessarily be the first step in the development of a science of enzyme actions and reactions.

Following this stage of the study, two methods of attacking the

problem present themselves. Enzymes, as catalysts, modify reaction velocities. A systematic study of reaction velocities, their significance, and the factors upon which they depend would be in order. Then the general problem of catalysts would be considered, and finally, enzymes as a special group of catalytic substances. The second method of attack would include the study of the chemical nature of enzymes, their compositions, structural formulas, and reactions. That is to say, enzymes would be studied as though they possessed definite chemical structures.

An attempt will be made here to indicate the progress which has been made in enzyme studies in recent years. The descriptive methods have shown so many reactions which are catalyzed by enzymes, and also such a great variety of animal and vegetable products which are capable of increasing the velocities of chemical changes, that reference to the larger textbooks of biological chemistry and to compilations of enzyme actions may suffice for the details of the older work in this field. A recent investigation of certain enzyme actions of various tissue and tumor extracts, more quantitative in nature than most of the earlier work will, however, be given in this connection. The newer methods of study, involving in the main the relations of enzyme actions and reactions to phenomena which have been studied in other fields of chemistry, will be taken up more in detail.

Although a great many reactions have been found to belong to the group of enzyme reactions, it is possible to classify them in a comparatively simple manner with the aid of some recent conceptions of theoretical chemistry. These theoretical views, involving the structures of chemical compounds, will be presented here as a necessary foundation for the consideration of enzyme reactions and the factors influencing their velocities.

Practically all enzyme actions deal with reactions of organic compounds. For a number of years, perhaps from 1870 or thereabouts until comparatively recently, in the teaching and treatment of chemical substances, it has been customary to consider chemistry as divided into two branches, inorganic chemistry and organic chemistry. Different methods were used in experimental work in handling and working with the substances grouped in this way, and as a result, theories were developed for one or the other of these divisions. These theories were apparently limited to one group.

For example, the theory of electrolytic dissociation in solution was not considered by some of its most active advocates to be applicable to organic compounds, while at the same time a number of organic chemists ignored this theory not only in their own experimental work, but also in their teaching and writing, even when dealing with inorganic substances.

Fortunately, in recent years, the development of chemical theory has taken a more rational trend. Theories are considered to apply to all chemical compounds. Division of substances into organic and inorganic compounds is for convenience only. This matter of convenience may refer to such practical questions as teaching, occurrence in nature, like properties, etc. A certain theory may be developed with one group of compounds. This theory apparently does not apply in the same way to a different class of compounds, but a more careful study will always show that conditions of testing may be different; that a common truth applicable in some way to both classes is present; and therefore that the theory as developed was incomplete. Thus, the theory of electrolytic dissociation was developed in connection with certain properties of inorganic substances in aqueous solution. At first, the theory was not considered to apply to organic substances either as solutes or solvents. Then further experimentation showed that organic substances, to varying extents, showed the phenomenon of electrolytic dissociation in solutions. The most recent development brings out the view that all chemical combinations between atoms are electrical in character, and that for a substance to show electrolytic dissociation in solution, the participation of a solvent with certain definite properties is essential.

With this general viewpoint of chemical theory in mind, it will be possible to consider the basic theories of chemical reactions somewhat further. Some of the views relating to the structures, chemical and physical, of the substances involved in enzyme actions will be given in this chapter, while in the next chapter the theoretical relations involved in the changes or in the dynamic processes occurring will be developed.

Enzyme reactions, or the reactions whose velocities are observed to be increased by enzymes, may be divided into two general classes. In one class, the elements of water or of some similar compound may be added to or eliminated from the substance being changed;

in the other class, oxidation or reduction occurs. The first class includes such reactions as the hydrolyses of esters, of complex carbohydrates including di-, tri-, and polysaccharides, of urea, of amides, etc.

The second class of reactions mentioned, that including oxidation and reduction, may be considered somewhat more in detail in this connection. Oxidation or reduction refers in any given case to one or more definite atoms of a molecule and not to the molecule as a whole. With inorganic compounds, the view has been generally accepted that oxidation consists in an increase in the positive charge of the atom being oxidized or a decrease in the negative charge. Using the electron conception of valence, these views become simplified and applicable to all classes of compounds. The electron conception of valence states that in the formation of a union or linking between two atoms, one of these atoms loses a (negative) electron and the other gains this electron. As a result of this linking, the first of these atoms acquires a unit positive charge, the second a unit negative charge. This means simply that valence instead of being denoted by a number is stated as a positive or negative number. In oxidation, the valence of the atom being oxidized is increased, in reduction, decreased. Thus, the change of ferrous chloride into ferric chloride is represented as follows:

$$\overset{++}{Fe}\overset{-}{Cl_2} \rightarrow \overset{+++}{Fe}\overset{-}{Cl_3}$$

The valence of the iron is increased one unit, the valence of the chlorine remains unchanged. The change from hydrogen sulfide to sulfur dioxide is represented as follows:

$$\overset{+}{H_2}\overset{--}{S} \rightarrow \overset{++++}{S}\overset{--}{O_2}$$

Here the valence of the sulfur is increased six units. Many other examples might be quoted but the significant feature is that one definite atom is oxidized or reduced, loses or gains negative charges. If the complete equation of a chemical reaction is written, then if one atom is reduced, some other atom must be oxidized or vice versa. It is necessary to have an equivalence of positive and negative charges or valences just as it is necessary to have an equivalence of atoms. The charges indicated as the valences of the atoms are only in special cases susceptible to direct experimental

measurement. They are assumed to be present as a consequence of the development of valence theories and account satisfactorily for a number of facts not readily explicable without them. The same views and structures apply to organic compounds. The change of methane to carbon dioxide may be represented as follows:

$$\overset{-4\ +}{C}H_4 + 2O_2 = \overset{+4\ =}{C}O_2 + 2H_2\overset{+\ =}{O}$$

(the oxygen molecule may be assumed here to be either $\overset{\cdot\cdot}{O} : \overset{=}{O}$ or $\overset{\cdot}{\overset{\cdot}{O}} : \overset{\cdot}{\overset{\cdot}{O}}$). The carbon atom is oxidized eight units of valence, the hydrogen is neither oxidized nor reduced, while the oxygen of the two oxygen molecules must be reduced eight units of valence. All organic oxidations and reductions may be considered similarly and bring such reactions into line with inorganic oxidations and reductions. This substitutes one explanation of oxidation-reduction phenomena for the two or more which were necessary when a fundamental distinction was made between the theories of structures of organic and inorganic compounds. With more or less complex organic compounds, a difficulty is met with at times in determining the atom or atoms which are oxidized or reduced, but even here, the general principles may be applied.

For the reactions of the first class involving in the main hydrolyses, the use of the electron conception of valence as described does not, at present, add anything essential to their consideration. There is no change in the state of oxidation of any of the atoms of the molecules taking part in the reactions.

The newer developments in the views of the structure of matter based upon the electronic configurations of atoms and molecules mark a definite advance in the treatment of these problems. There is not as yet general agreement with reference to the solutions of the problems.

Two general theories or types of relations are under discussion at the present time. In the one, the electrons are assumed to be fixed in more or less definite positions in the atom or molecule; in the other, they are assumed to be describing orbital motions. The former theory was developed mainly by J. J. Thomson,[1] Lewis,[2]

[1] For the latest statement of the views of J. J. Thomson cf. "The Electron in Chemistry," published by the Franklin Institute, Philadelphia, 1923.

[2] G. N. Lewis, *Jour. Amer. Chem. Soc.*, 38, 762 (1916); "Valence and the Structure of Atoms and Molecules"; American Chemical Society, Monograph Series. The Chemical Catalog Co., Inc., New York, 1923.

Kossel,[3] and Langmuir [4]; the latter is generally connected with the names of Bohr [5] and Rutherford.[6]

In the so-called "static" atom, electrons are assumed to surround the positive nucleus of the atom arranged in concentric groups or shells. The number of such electrons is equal to the positive charge of the nucleus (number of protons minus number of electrons) or the atomic number. In any atom, the arrangement is such that two electrons are in the first group or shell, eight in the second, eight in the third, and so on, or less in the outermost shell with a smaller or insufficient number of electrons. With a three dimensional configuration, the greatest stability is found in the symmetrical structures in which a complete set of eight electrons are arranged at the corners of a cube. Complete symmetrical structures are found in the rare gases; helium with a group of two electrons, neon with groups of two and eight, argon, two, eight and eight, and so on. Other atoms, containing a smaller number of electrons in the outer shell, tend to give off or take up electrons to form a complete shell. This giving off and taking up of electrons by two atoms results in the formation of chemical compounds. The production of symmetrical and stable electron configurations in which one or more electrons of two different atoms take part is thus seen to be the characteristic feature of chemical combination. The formation of the stable group of eight electrons is the main tendency which is shown. A general rule "which includes the possibility of complete ionization as a special case" is given by Lewis (p. 79). "Two atoms may conform to the rule of eight, or the octet rule, not only by the transfer of electrons from one atom to another, but also by sharing one or more pairs of electrons. These electrons which are held in common by two atoms may be considered to belong to the outer shells of both atoms."

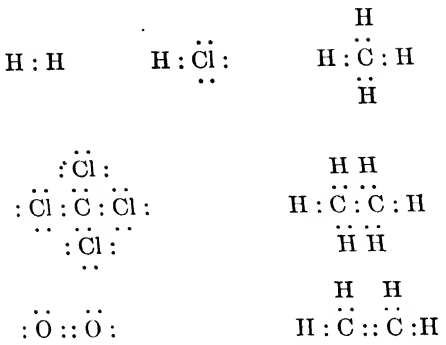
A few examples (necessarily in two dimensions) may indicate the nature of the structures for simple substances:

[3] W. Kossel, *Ann. der Phys.*, *49*, 229 (1916).

[4] I. Langmuir, *Jour. Amer. Chem. Soc.*, *38*, 222 (1916); *41*, 868, 1543 (1919); *42*, 274 (1920).

[5] N. Bohr, *Phil. Mag.* (6) *26*, 1857 (1913). Reviews and summaries which may be mentioned in this connection are the following: N. Bohr, *Nature*, *112*, 29 (1923); *Die Naturwissenschaften*, *11*, 533 (1923); E. E. Free, *Ind. Eng. Chem.*, *16*, 102 (1924); etc.

[6] Cf. especially E. Rutherford, Presidential address, "The Electrical Structure of Matter," British Association for the Advancement of Science, Liverpool, 1923.



Only the electrons in the outer shell (the so-called valence electrons) are shown. For the usual double bond, two pairs of electrons are shared so as to make up a complete set of eight electrons for each atom. This is shown, not altogether satisfactorily, in the structures for oxygen and ethylene.

Although it is not possible to enter into any detail with these views, their nature and trend may be sufficiently evident. They are proving extremely useful not only as a classifying principle, but also in developing new lines of work. For example, W. A. Noyes [7] recently showed that the mechanism of addition reactions could be readily explained on the basis of a pair of electrons held in common by two atoms forming a bond, and further that these views were not in conflict with, but represented a definite advance on, the older positive—negative theories of chemical combination.

It may be pointed out that in the development of J. J. Thomson, a law of force, modified somewhat from the customary one, was used in connection with the various electron configurations, and the various applications to chemical relations based upon these.

The Bohr-Rutherford theory of atomic structure based in the first instance upon the emission of spectral lines and their relations to each other is apparently fundamentally different from the theory of the structure of the static atom.

For the hydrogen atom consisting of a proton and an electron, Bohr showed that the electron could move in a number of stable orbits controlled by the attractive force of the nucleus, without

[7] W. A. Noyes, *Jour. Amer. Chem. Soc.*, *45*, 2959 (1923).

losing energy by radiation. (Rutherford, p. 12.) "The position and character of these orbits were defined by certain quantum relations depending on one or more whole numbers. It was assumed that radiation was only emitted when the electron for some reason was transferred from one stable orbit to another of lower energy. In such a case it was supposed that a homogeneous radiation was emitted of frequency ν determined by the quantum relation $E = h\nu$ where E was the difference of the energy of the electron in the two orbits. Some of these possible orbits are circular, others elliptical, with the nucleus as a focus. . . . In a radiating gas giving the complete hydrogen spectrum there will be present many different kinds of hydrogen atoms, in each of which the electron describes one of the possible orbits specified by the theory. On this view it is seen that the variety of modes of vibration of the hydrogen atom is ascribed, not to complexity of structure of the atom, but to the variety of stable orbits which an electron may occupy relative to the nucleus. This novel theory of the origin of spectra has been developed so as to apply not only to hydrogen but to all the elements. . . . The problem is obviously much less complicated for hydrogen than for a heavy atom, where each of the large number of electrons present acts on the other, and where the orbits described are much more intricate than the orbit of the single electron in hydrogen. Notwithstanding the great difficulties of such a complicated system of electrons in motion, it has been possible to fix the quantum number that characterizes the motion of each electron, and to form at any rate a rough idea of the character of the orbit."

"It may be of interest to try to visualize the conception of the atom we have so far reached by taking for illustration the heaviest atom, uranium. At the center of the atom is a minute nucleus surrounded by a swirling group of 92 electrons, all in motion in definite orbits, and occupying but by no means filling a volume very large compared with that of the nucleus. Some of the electrons describe nearly circular orbits round the nucleus; others, orbits of a more elliptical shape whose axes rotate rapidly round the nucleus. The motion of the electrons in the different groups is not necessarily confined to a definite region of the atom, but the electrons of one group may penetrate deeply into the region mainly occupied by another group, thus giving a type of interconnection or coupling between the various groups. The maximum speed of any electron

depends on the closeness of the approach to the nucleus, but the outermost electron will have a minimum speed of more than 1,000 kilometers per second, while the innermost K electrons have an average speed of more than 150,000 kilometers per second, or half the speed of light. When we visualize the extraordinary complexity of the electronic system we may be surprised that it has been possible to find any order in the apparent medley of motions." (Rutherford, p. 13.)

The Bohr atom appears to be based upon more satisfactory theoretical grounds than the static atom. On the other hand, the static atom has proven to be more generally and readily applicable to chemical structures and to permit of a comparatively ready assimilation with the view of "chemical bonds." At the same time, the striking success which the Bohr atom has had in certain fields of chemistry, such as, for example, in predicting the properties of the missing element, number 72, later called hafnium and because of the incentive given, resulting in its discovery, must not be overlooked.

Attempts to unite the two views of atomic structure, have not, up to the present, appeared to be satisfactory.

In this brief, and necessarily inadequate, outline, obviously no attempt was made to show the development of the various views. Only rough cross-sections, so to speak, have been given. It is hoped, however, that a view has been obtained which shows the trend the work on chemical structures is taking, based upon the more fundamental units of chemical composition, the electron and the proton.

These views of chemical structure are apparently not necessary in a treatment of enzyme reactions. While it is true that enzyme reactions might be treated by themselves without considering their relation to, or bearing on, other chemical phenomena, it would appear that the time has come to include enzyme reactions in the group of general chemical reactions, amenable to the same conditions and explainable by the same theories. Any general theory found useful in the consideration of other chemical reactions, should therefore be considered or at least mentioned in connection with enzyme reactions. The further development of enzyme actions and reactions will unquestionably follow chemical lines. The science of chemical structures and reactions is at present based upon certain theories, and the study of such structures and reactions involves the application of these theories and at the same time results in the further

development of these theories. There is no reason to place enzymes and their actions in a separate category. As chemical substances and chemical actions they must form a part of the chemical whole. For various reasons, the application of comparatively simple chemical relations to these actions has not been as successful heretofore as with some of the other groups of chemical substances and reactions. However, this does not make further attempts in the same direction unnecessary. It is therefore advisable to present some of the more recent viewpoints of chemical theory bearing upon these and related problems, with the hope and expectation that they will be found applicable and useful in some form. Especially in the oxidation reactions of enzymes, as will be brought out again in a later chapter, are the results at present available uncoördinated and unsystematized.

Several other theories and viewpoints must also be discussed briefly since the consideration of chemical actions is based to a certain extent upon them and their modifications.

In the first place the question of solution may be considered, since most enzyme actions take place in the presence of water, either in aqueous solution, frequently colloidal, or in aqueous suspension.

The ionic theory or the electrolytic dissociation theory of Arrhenius has been extremely successful in accounting for a number of facts observed in solutions and in correlating apparently diverse phenomena. The reactions of salts in aqueous solutions were ascribed to the presence of ions whose existence has been demonstrated repeatedly. Some zealous supporters of the ionic theory even went so far as to state that only ions take part in chemical reactions. This point of view was never considered seriously. The fact that unionized molecules as well as ions may take part in chemical reactions and that frequently both may be assumed to do so, was shown later by a number of workers. This question will be taken up in greater detail in Chapters II and III.

An attempt was made some years ago to develop these problems further.[8] A somewhat more detailed account was published later.[9] In brief, the view was advanced that the readiness or speed with which reactions occurred was a phenomenon not dependent upon

[8] K. G. Falk and J. M. Nelson, *Jour. Amer. Chem. Soc.*, *37*, 1732 (1915).

[9] K. G. Falk, "Chemical Reactions; Their Theory and Mechanism," published by the D. Van Nostrand Company, New York, 1920.

the existence of ions. The occurrence, existence, and stability of ions in the same way had nothing to do directly with the occurrence of chemical reactions. There is, however, an indirect connection. The physical property shown by the ability to conduct the electric current in solution and the chemical property shown by the ability and readiness to undergo change in composition alone or in conjunction with other substances, are both assumed to be due to the same underlying cause. This cause, while producing both effects, need not produce both quantitatively at the same rate. That is to say, under certain conditions, the physical property would be much the more marked and amenable to experiment; under other conditions, the chemical. Elsewhere the general theory was suggested and developed that chemical reactions between two or more substances depend upon the primary formation of addition compounds. This view will be spoken of more in detail in Chapter II. In the present connection it may be pointed out that it is probable that in solution, the property of the solvent of forming addition compounds with the dissolved substances is the common cause of the two sets of phenomena, physical and chemical. In aqueous solutions, compounds of the nature of hydrates, which have been shown to exist in a number of cases, may well be the cause. In some cases, such as with uni-univalent salts in aqueous solutions, very close parallelism exists between the physical and chemical properties; with uni-divalent and more complex salts, the parallelism is not obvious or does not exist at all. Quantitative proof of this theory is not at hand, but it has been found useful in the consideration of reactions and will be used here. To sum up these relations: The changes occurring in chemical reactions do not depend upon the electrolytic dissociation of the reacting substances. The chemical changes are accompanied very often by electrolytic dissociation phenomena, but the latter parallel the former (or vice versa) and do not necessarily precede or cause them. The modern views of atomic structure assume various arrangements of electric charges (electrons and protons) in the atoms, while in the molecules made up of atoms in combinations, the distributions or arrangements of certain of these electrons have been changed. The experimental facts of electrolytic dissociation offer a method for making some of these electric charges, due to the change in electron distribution or arrangement, susceptible to measurement, but electrolytic dissociation does not produce these

charges on the ions. This point must be clearly understood, otherwise confusion will result.

The hydrogen ion concentration has been much emphasized in connection with enzyme actions. At the same time, its importance in other chemical actions may have been overlooked. In aqueous solutions it is unquestionably one of the most important standards by which the properties of the solvent and dissolved substances may be controlled, and it is possible that it is from this point of view that this factor should be considered.

Another set of phenomena which has given rise to much discussion especially in recent years is included under the general topic of "colloid chemistry." Colloids are obviously of importance in connection with the study of substances obtained from living matter. The point of view which will be adopted here is that colloids as chemical substances will be considered in the same way as other substances, and that colloidal solutions will be assumed to possess chemical properties fundamentally similar to those of other solutions. If apparent exceptions occur, or if phenomena predominate which are more in the background with the noncolloidal substances and solutions whose relations have been studied over a longer period of time, these exceptions and phenomena will not be designated by new names or branches of science, but will be retained under the group of relations which have not as yet been developed satisfactorily and which require further quantitative study. "Colloid chemical treatment of certain phenomena is often regarded as contradictory to e.g. the pure chemical or the electro-chemical treatment. We often hear people speak of the colloid chemical theory of adsorption in contradistinction to the purely chemical theory; and of the colloid chemical theory of proteins in contradistinction to the chemical or the electro-chemical theory of proteins. To a certain extent this is only a quibble about words and terms. Traced to their very sources, all forces chemical, colloid-chemical and physical are electrical in nature and therefore both physics and chemistry deal with the same forces—the attractions and repulsions between electrons and positive nuclei. This does not hinder us from dividing the vast field into practical domains of cultivation according to methods used for the work. Colloid chemistry is such a domain. The colloid chemist must not forget, however, that his science does not operate with any specific forces or substances and that the

colloid phenomena traced back to their sources are built up of ordinary physical and chemical phenomena. So for instance, it is not admissible to regard the degree of dispersion in a colloid system as an arbitrary property determining the system, but it is of importance because it forces certain conditions upon the system." [10]

Similarly, adsorption compounds will be considered to be fundamentally chemical in character. Because of such facts as amount of surface involved and physical state of subdivision of the reacting components, the laws of definite and multiple proportions cannot in most cases be proven to apply to the masses in question, and equilibrium between all of the various components may not be attained in many cases. The evidence which is rapidly accumulating indicates that chemical compounds are formed with the limitations as to their extent just referred to, and that the same general laws of combination exist here as with other chemical compounds. Another factor which will be referred to again presently depends upon the selective penetration of membranes or outer surfaces of colloidal and other particles by diffusible and partially diffusible substances. These "membrane equilibria" or distribution of substances between solutions separated by partially permeable membranes have been studied extensively in recent years.

Before taking up these questions, reference may be made to some recent work by Langmuir and by Harkins on the orientation of molecules in surface films. The point of view may be shown best perhaps by a quotation from a paper by the latter: [11] "In this paper data will be presented for the work done when the surfaces of two liquids come together to form an interface. The numerical value of this work in ergs per square centimeter is characteristic of each class of compounds, and the data show in a very striking manner that the film of any liquid in contact with water is composed of molecules oriented so that the active (or polar) group at the end of any hydrocarbon chain is in contact with the water. . . . It will be shown that the attraction between water and another liquid is one of the important factors in the determination of the solubility of the other liquid in water." The groups most active toward the

[10] T. Svedberg, "Colloid Chemistry," American Chemical Society, Monograph Series. The Chemical Catalog Company, Inc., New York; 1924; pp. 11-12.

[11] Cf. W. D. Harkins and co-workers, *Jour. Amer. Chem. Soc.*, *39*, 354, 541 (1917); *41*, 970 (1919); *42*, 700 (1920).

water surface, or, as Harkins calls them, the very polar groups, were shown to be CO_2H, CO, CN, OH, and $CONH_2$.

The conclusions of Langmuir [12] are essentially the same except that he speaks of chemical combination between certain of the groupings or atoms of the substances composing the surfaces. Although he considers primary and secondary valences, in a sense following Werner, and is not entirely clear as to these, the crux of his theory is perfectly definite and may be given best in his own words: [13] "From the viewpoint adopted in the present paper the forces involved in adsorption, surface tension, etc., are strictly chemical in nature, that is, they do not differ in any essential respect from the forces causing the formation of typical chemical compounds." [14]

These views are presented as they show the trend which the studies on adsorption and surface actions are taking. The general conclusion appears to be that the forces acting are chemical in character and must be treated in the same way as other chemical actions. To the organic chemist, especially, the results of Langmuir and of Harkins are of interest from another point of view. Those groups which are found to be most active in adsorption and surface phenomena, and combine chemically in such surfaces, are the same groups which the organic chemist has found to act most readily with chemical reagents. The organic chemist is able to bring about chemical reactions with specific atoms or groups of atoms in a more or less complex organic molecule by a suitable choice of reagents and conditions. The physical chemist has now shown how to measure by purely physical means the action of an atom or a group of atoms in a complex organic molecule. The orientation by means of which it has been made possible to make these measurements (of surface tension) is based upon a chemical reaction of the atom or group of atoms in question with the second surface and is based therefore upon the same underlying properties which the organic chemist studies when he determines the "reactivity" of a grouping. The active groups of the physical chemist and of the organic chemist are the same, and this method of determining such groups promises to be of value in the future study of organic compounds, whether

[12] I. Langmuir, *Met. Chem. Eng.* *15*, 468 (1916); *Jour. Amer. Chem. Soc.* *39*, 2221 (1916); *39*, 1848 (1917); *40*, 1361 (1918).

[13] I. Langmuir, *Jour. Amer. Chem. Soc.*, *39*, 1848 (1917).

[14] Cf. also N. K. Adam, *Proc. Roy. Soc., London* (*A*) *99*, 336 (1921); *101*, 452, 516 (1922); *103*, 676, 687 (1923).

these be simple in composition, or of complex nature such as are present frequently in biological material.

So far molecular and atomic structures and certain surface structure have been considered. In treating enzymes and enzyme actions, their occurrence in biological material makes it advisable to mention biological structures. Here the cell may be considered the unit. The study of this unit has thrown much light on its modes of action and properties. "The evidence from every source demonstrates that the cell is a complex organism, a microcosm, a *living system*. With the microscope we distinguish in this system a clear and apparently structureless ground-substance or *hyaloplasm* in which are suspended a great variety of visibly different formed bodies, widely diverse in form and function, each of which plays its own particular part in the activities of the system. Examples of these bodies are, first of all, the nucleus, and then the cytoplasmic chondriosomes and plastids, the Golgi-bodies and central bodies and many kinds of granules and fibrillæ. The functions of these various bodies are still imperfectly known; but all undoubtedly are centers of specific chemical activities which contribute in one way or another to the life of the cell."[15] . . . "the cytologist finds reason enough to exercise his wits upon the apparently structureless ground-substance or hyaloplasm that seems to constitute the fundamental basis of protoplasm and to be the source of many of its formed elements. He cannot resist the evidence that the appearance of a simple, homogeneous colloidal substance that is offered by the hyaloplasm is deceptive; that it is in reality a complex, heterogeneous or polyphasic system. He finds it difficult to escape the conclusion, therefore, that the visible and the invisible components of the protoplasmic system differ only in their size and degree of dispersion; that they belong to a single continuous series; and that the visible structure of protoplasm may thus give us something like a rough magnified picture of the invisible.[16]

One of the questions which is involved in the life processes of cells includes the entrance into the cell of substances necessary for the continuance of the life process and the elimination of unnecessary and undesirable substances. This question involves the passage of

[15] E. B. Wilson, "The Physical Basis of Life," Yale University Press, New Haven, 1923, pp. 4-5.

[16] E. B. Wilson, p. 28.

substances through membranes which may or may not be permeable to a given substance or constituent of such a substance. Much valuable work has been done in the past years on this phenomenon by J. Loeb [17] and the problem presented clearly with much evidence looking toward a solution of certain phases of it.

The question of "membrane equilibria" was submitted to study. In these equilibria the membrane in question is permeable to one component of a mixture or to one ion constituent of an ionized molecule (which may be a protein salt). The distribution of the two ions, or components, or salt constituents, in the solutions separated by the membrane in the presence of other salts, acids, or bases, with or without an ion or salt constituent in common, was calculated on thermodynamic principles in 1875 by J. Willard Gibbs [18] and later by Donnan [19] who has also carried out extensive investigations along this line. The thermodynamic conclusions were found to be borne out fully by the results, thus making these structures and the part they play in influencing chemical change, susceptible to experiments based upon firm theoretical grounds. The conclusions were found not to be limited to structures in which artificial membranes such as the surfaces of collodion bags were used, but could be carried over with most satisfactory results to colloidal particles where the outer surface of the particle could be considered to act as the membrane. For instance, the state of a (colloidal) particle of casein in acid solution is a case in point. The hydrogen ion concentration of the particle may be different from that of the surrounding solution because of the distribution of the hydrogen ion, which can pass readily through the outer surface of the particle, and of the membrane equilibrium which is thus produced.

These facts add greatly to the significance of the findings and show a connection between the results obtained with colloidal particles ordinarily dealt with and the phenomena occurring with cells. They are specially significant in a study of enzyme actions for enzymes occur almost exclusively in cells. Their actions in living matter are therefore closely related to, if not dependent upon, the passage of substances through cell walls; while their study in the

[17] J. Loeb, "Proteins and the Theory of Colloidal Behavior."

[18] "The Scientific Papers of J. Willard Gibbs," Vol. 1, p. 83. Cf. also G. S. Adair, *Science 58*, 13 (1923).

[19] F. G. Donnan, *Z. Elektrochem. 17*, 572 (1911).

laboratory involves in many cases their removal, by extraction or otherwise, from the cells.

In emphasizing the importance of these relations, it must not be forgotten that substances in living matter, and especially the changes occurring with and in cells, are not as a rule in thermodynamic equilibrium with their environment. It is necessary to call attention to this fact, since the impression must not be created that the problem is solved in any way. A definite beginning has been made, valuable data have been obtained, and a road to further progress has been indicated.

Crystal structure has not been mentioned heretofore in this chapter. The extensive literature which has accumulated in the last years dealing with the arrangement of atoms in crystals as determined by their x-ray spectra, can only be referred to here. Space cannot be devoted to these interesting and important results, not because in the development of structure views in nature they are less significant than any of the theories which are described, but because, as far as can be seen at present, the application of the methods of working and the conclusions reached are not as directly and immediately applicable to the materials and substances which are met with in enzyme studies.

If any general viewpoint may have been reached in this brief and necessarily incomplete survey of structures of atoms, molecules, and more complex materials, it would consist in the fact that the studies in progress are continually indicating the complexity of units which up to recently have been considered more or less homogeneous. Actions and phenomena are being more and more localized in definite parts of atoms, of molecules, of cells. At the same time that the responsibility for certain definite phenomena are fixed in this way, the rest of the structure, while apparently not taking part in the action, must also be influenced or modified to an extent which varies with the conditions, and which will inevitably manifest itself in some subsequent action.

II.—Velocities of Chemical Reactions

An attempt will be made in this chapter to indicate the place which enzyme actions and reactions may be said to occupy in relation to the general problem of chemical change. Some of the more important viewpoints which are under discussion at the present time with reference to the chemical and physical structures, from electronic to colloidal, of the substances which may be involved in one way or another, were presented in the preceding chapter. These structures, as such, do not come into consideration in the subject matter to be presented in this chapter. It is obvious, of course, that for the most satisfactory understanding of the various relations involved, all possible viewpoints must be considered simultaneously, even if, for convenience of presentation, they may be given separately.

The actions of enzymes manifest themselves in general by changes in the velocities of certain chemical reactions. Before entering into the specific enzyme actions, it would be well to review what is meant by the velocity of a chemical reaction, the formulations and equations which are used, the significance of the various terms of such equations, the factors which may limit such formulations, and the chemical conclusions which may be derived from kinetic considerations.

The kinetic developments have been based in the main upon the number of different molecules which react or are changed in unit time in the reaction under consideration. Thus the mathematical expressions which are derived depend upon whether one molecule is undergoing change as in a "monomolecular" reaction, whether two as in a "bimolecular" reaction, three as in a "termolecular" reaction, etc. Such expressions evidently depend upon a knowledge of the chemical equation of the given reaction. While this is true to a certain extent, deductions from the mathematical expressions may show that the chemical equation does not represent the change as it occurs; that is to say, either the mathematical equation or the

chemical equation or both are incomplete. Light has been thrown on the mechanisms of a number of reactions in this manner.

It is obviously impracticable to give here a complete review of the subject of chemical kinetics. Only some of the salient features and those which may be of more direct interest in the present connection will be considered.

The law of mass action forms the basis of the exact study of chemical kinetics. This law states that the amount of substance undergoing change in a unit of time is proportional to the active mass present during that time. This law is of general applicability. In applying it in chemical reactions, it is obviously necessary to use certain units of mass or quantity in order to express the active mass of substance present at any given time. The simplest view to take is that the active mass of a substance is given by its molecular concentration. For practical purposes, therefore, the number of gram molecules, or mols per liter of volume, is used as the active mass.

Before going farther, however, it is necessary to emphasize the simplification which has been introduced. The active masses have been replaced by molecular concentrations, and therefore the law of mass action has been changed to the law of concentration action. If, now, deductions from the law of concentrations are found not to be valid, this does not mean that the law of mass action does not hold, but that an incorrect hypothesis may have been introduced in the substitution of concentrations for active masses.

In the simplest case in which one substance is undergoing change, if the initial concentration of this substance is denoted by a, and at the end of the time t, x gram molecules of a have been transformed, $a - x$ remaining unchanged, then the law of concentration action requires that, the temperature remaining constant,

$$\frac{dx}{dt} = k\,(a - x), \qquad (1).$$

By integration and evaluation of the integration constant by putting $t = 0$,

$$k = \frac{1}{t} \log_e \frac{a}{a - x}, \qquad (2)$$

or when x_1 and x_2 denote the amounts of substances transformed after time intervals t_1 and t_2,

$$k = \frac{1}{t_2 - t_1} \log_e \frac{a - x_1}{a - x_2} \qquad (3).$$

These equations for a monomolecular reaction have been applied to a number of reactions and a constancy of k demonstrated. For example, in the transformation of dianthracene ($C_{28}H_{20}$) into anthracene ($C_{14}H_{10}$) according to the chemical equation

$$C_{28}H_{20} = 2\,C_{14}H_{10}$$

the following results were obtained at 152° in phenetol as solvent: [1]

t	*0*	*865*	*1225*	*2345*	*3845*	*5310*
D	*16.38*	*9.35*	*7.03*	*3.26*	*1.18*	*0.55*
kx10^4		1.2	1.3	1.3	1.3	1.2

In these results, t represents the time in minutes from the beginning of the reaction, D the milligrams per liter of dianthracene present at time t, and kx10^4 the monomolecular reaction velocity constant as calculated by means of equation (3) from each pair of determinations. It is evident that the value of the constant shows that the reaction follows the indicated law.

Equation (2) may also be written in the form

$$\frac{a - x}{a} = e^{-kt},$$

or the substance is transformed at a rate which varies in an exponential manner with the time.

Now k in these equations represents a constant characteristic for the reaction being measured under the given conditions. Its physical significance is the rate of transformation of unit concentration of the substance (from equation (1)). Its numerical value is independent of the original concentration (a) of the substance undergoing change.

Several further derivations from equation (2) have been made and are in use at present mainly with radioactive changes. The term $1/k$ may be called the period of average existence of the substance undergoing change and denotes the time which would be required for the substance to be transformed completely if the same amount of substance being transformed at the instant in question continued to be transformed.

[1] R. Luther and F. Weigert, *Sitz.-ber. Kgl. pr. Akad. Wiss. Berlin, 1904*, 828.

The period of half change, or time required for half of the substance to be transformed, is found to be

$$t = 0.6932 \frac{1}{k}$$

This period of time will be the same, independent of the amount of substance with which the calculation is made. It is therefore a useful constant for reactions obeying the monomolecular law.

The chemical changes involved in monomolecular reactions lead to some suggestive possibilities with regard to the reacting substances. In these reactions, only one substance is undergoing change. It follows, since the whole amount of substance is not transformed instantaneously, that the substance must be present in different states or conditions. Various attempts have been made to account for these differences. A number of these were reviewed in another connection.[2] As it would lead too far to repeat these, only the statement of the views which appear to have been generally accepted will be quoted: "The cause of the monomolecular reaction velocity law is to be found in the electronic arrangements in the atoms and molecules and the changes in these arrangements due to certain vibratory motions; certain configurations are responsible for the occurrence of chemical changes (or electronic rearrangements), the frequency of the occurrence of these configurations obeying some law of probability; radiant energy may play an important part in bringing about certain configurations and changes in configurations, but radiant energy in bringing about such configurations and changes is not absorbed according to the simple Einstein law of photochemical equivalence."

In a bimolecular reaction two substances are undergoing change simultaneously. Since it is probable that the amount of reaction is proportional to the number of times the particles of the two substances meet, the total change at any instant would be proportional to the product of the concentrations (or active masses) of the substances undergoing changes. This formulation includes the relation described with monomolecular reactions, namely, that the molecules

[2] K. G. Falk, "Catalytic Action," The Chemical Catalog Company, Inc., New York, 1923, pp. 82-93.

in addition to meeting must also be in a suitable state or condition for reaction to occur. Methods of formulation similar to those given for monomolecular reactions are in use. Starting with a gram molecules of each of the reacting substances, if after time t, x gram molecules of each have reacted, then the velocity of the reaction will be represented by the equation

$$\frac{dx}{dt} = k\,(a - x)^2 \tag{4}$$

which, on integration, becomes

$$ak = \frac{1}{t} \cdot \frac{x}{a - x} \tag{5}$$

in which ak is constant.

Starting with different amounts, a and b, of the two products, the equations assume the following forms:

$$\frac{dx}{dt} = k(a - x)\ (b - x) \tag{6}$$

$$(a - b)k = \frac{1}{t}\log_e \frac{a(b - x)}{b(a - x)}. \tag{7}$$

The usual example of a bimolecular reaction is the hydrolysis of ethyl acetate by sodium hydroxide:

$$CH_3CO_2C_2H_5 + NaOH = CH_3CO_2Na + C_2H_5OH.$$

With equivalent amounts of reacting substances, the following results have been obtained (equation 5) and are generally quoted: [3]

t	x	ak
5	5.76	0.113
15	9.87	0.107
25	11.68	0.108
35	12.59	0.106
55	13.69	0.108
120	14.90	0.113

With amounts of reacting substances not equivalent, the following results may be quoted [4] (equation 7):

[3] R. B. Warder, *Ber.*, *14*, 1361 (1881); *Amer. Chem. Jour.*, *3*, 340 (1882).
[4] L. T. Reicher, *Lieb. Ann.*, *228*, 257 (1885); *232*, 103 (1886).

Alkali in Excess				*Ester in Excess*			
t	$(a-x)$	$(b-x)$	$(a-b)k$	t	$(a-x)$	$(b-x)$	$(a-b)k$
0	0.5638	0.3114	.....	0	0.3910	0.6593	.....
393	0.4866	0.2342	0.0335	342	0.2885	0.5568	0.0346
669	0.4467	0.1943	0.0342	670	0.2239	(0.4222)	0.0347
1010	0.4113	0.1589	0.0339	888	0.1925	0.4605	0.0345
1265	0.3879	0.1354	0.0346	1103	0.1677	0.4350	0.0344

These examples will suffice to show the use of the equations, although many other reactions have been found to follow the bimolecular law.

The experimental results which have been given to illustrate mono- and bimolecular reactions show that the kinetic laws hold for these cases. A number of further reactions which might be quoted show in the same way the apparent validity of the deductions. However, to assume that the kinetic laws hold for every reaction under varying conditions is not permissible. In fact, it is not even permissible to extend the relations for any one reaction beyond the limits where the law has been found experimentally to hold. Increasing the accuracy of the experimental work and also modifying the conditions, for example by the addition of a so-called neutral salt, has resulted in several cases in changing the value of the constant, which had under different conditions shown constancy, so that the kinetic equations could no longer be used satisfactorily. This question will be taken up again in Chapter III. For the present, it may be stated that when satisfactory values for the velocity constants have been obtained, these values should not, without further evidence, be applied to the reactions under modified conditions, unless a careful scrutiny is made of the changes in the conditions and the influences these conditions exert upon the substances and their concentrations, whether these be calculated as molecules or ions or in some other way. It is possible that at times assumptions have been introduced into the calculations in this way, and that the apparent lack of agreement of the kinetic with the chemical equations is due to incorrect designation of the molecular species or their concentrations which are involved in the reaction.

With three substances taking part in the reaction, starting with the same initial concentrations, the equations assume the following forms:

$$\frac{dx}{dt} = k(a - x)^3 \tag{8}$$

$$k = \frac{1}{t} \cdot \frac{1}{2}\left(\frac{1}{(a - x)^2} - \frac{1}{a^2}\right). \tag{9}$$

Here k is inversely proportional to the squares of the initial concentrations of the reacting substances.

In general, the equations for an n-molecular reaction are as follows:

$$\frac{dx}{dt} = k(a - x)^n \tag{10}$$

$$k = \frac{1}{t} \cdot \frac{1}{n - 1}\left(\frac{1}{(a - x)^{n-1}} - \frac{1}{a^{n-1}}\right). \tag{11}$$

The number of reactions which obey the equations of higher orders decreases with the increase in the number of substances taking part in the reactions. From kinetic considerations such a result would follow from the probability of the smaller number of meetings of a greater number of molecules with resulting reaction.

The equations which have been given form the basis for chemical kinetics. Since, in this book, it is intended only to outline such relations, the reader is referred to other works where these velocity equations are treated more in detail and more examples quoted. The methods which have been suggested for determining the order of a reaction will not be gone into here.

The further considerations will take up questions which have been found to be of interest in connection with enzyme and similar reactions.

The velocity equations refer in any given case to a definite constant temperature. Increase in temperature will increase the velocity of reaction or the value of k. It has been found experimentally that an increase of 10° in temperature will increase the value of k or the velocity of a chemical reaction two to three times at temperatures in the neighborhood of those used ordinarily. Empirical equations have been proposed for this large increase in velocity with rise in temperature, but up to the present there appears to be no generally satisfactory explanation for the phenomenon.

The value of k is characteristic for a given reaction under definite conditions at a definite temperature. Any change in the reaction

mechanism or the conditions under which the reaction is taking place would show itself in a changing value of k.

If one of the reacting substances is present in large excess so that in the chemical reaction taking place its concentration remains practically unchanged, then this concentration may be considered to be constant in the velocity equation and the order of the reaction found will be smaller than that actually occurring. Thus, in the hydrolysis of sucrose according to the equation

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6,$$

the velocity of the reaction would be expected to be of the second order. In dilute solution, the concentration of the water remains practically unchanged so that the velocity of the reaction may be found to be proportional to the concentration of the sucrose. This reaction is, therefore, often considered to be a monomolecular reaction.

The chemical reactions have so far been considered as proceeding in one direction only. As a matter of fact, few reactions in homogeneous media do this. The products formed tend to recombine to form the original substances with a velocity of their own. Similar velocity equations can therefore be set up to represent these reverse reactions. After a certain time, these reactions in opposite directions will proceed at such rates that the composition of the mixture will remain unchanged; that is, equilibrium will have been attained. This equilibrium will be kinetic, not static; reaction still continues, but in opposite directions so that the chemical composition of the mixture is constant. The kinetic equations to represent such an equilibrium will evidently consist of the difference in the velocities of the two reactions.

In equation (10), denoting the reverse reactions as follows:

$$\frac{dx}{dt} = k_1(a_1 - x)^{n_1} \qquad \text{and} \qquad \frac{dx}{dt} = k_2(a_2 - x)^{n_2}, \tag{12}$$

then for equilibrium conditions

$$k_1(a_1 - x)^{n_1} = k_2(a_2 - x)^{n_2}, \quad \text{or}$$

$$\frac{k_1}{k_2} = \frac{(a_2 - x)^{n_2}}{(a_1 - x)^{n_1}} = K, \tag{13}$$

in which K represents the equilibrium constant of the reaction at a definite temperature. Both k_1 and k_2 vary with the temperature

and not as a rule at the same rate; therefore K may also vary with the temperature, but need not increase as do the velocity constants with rise in temperature. This method of developing the significance of the equilibrium constant must retain in that term the limitations which are inherent in the velocity constants. The factor relating to the law of concentrations enters here. Another method of developing the equilibrium constant is from the thermodynamic side without using the concepts of reaction velocities. This method, however, has similar limitations in that the simple laws of dilute solutions (or of gases) are assumed to hold for each constituent.

Equation (13) brings out another fact. If a condition of the reaction in one direction is changed so as to increase its velocity, but at the same time does not change the value of the equilibrium constant, then the velocity of the reaction in the opposite direction must be increased correspondingly. This is of interest in connection with the question of catalysts.

At this point attention may be called to the fact that a comparison of the chemical reaction, chemical equation, and mathematical equation will throw light on many of the questionable points involved. Each of these concepts has been evolved on certain definite bases, and each possesses certain limitations. For example, the treatment of successive reactions for which some equations will be shown directly serves to illustrate this question. The use of a chemical equation to represent one or more of the steps of a series of successive reactions is largely a matter of choice or convenience and the mathematical equation will then, in addition to the limitations inherent in its nature, also contain the limitations included in the application of the chemical equation to the chemical reaction.

The three concepts, chemical reaction, chemical equation, and mathematical equation, are supposed to describe the same phenomenon in any given case. Actually they do so only as an ideal condition, and the possibility of deviation becomes greater with increasing complexity of the reactions and with decreasing care in the use of terms and expressions. The chemical equation representing a chemical reaction is, as a rule, definite in that a specific change or reaction is illustrated. The mathematical equations involve deductions from the chemical equation by the application of mathematical processes to terms which represent to a greater or less degree of approximation the chemical terms or substances in the

chemical equation. In these equations, first chemical and then mathematical, the changes considered are perfectly definite. In an actual chemical reaction, the conditions are hardly ever such that the chemical and mathematical equations denote the complete condition of affairs. Generally more than one change is possible in the chemical reaction and the chemical equation may represent only the change in which the worker is interested. Other changes which may occur are ignored. Also, substances which take part, but whose final compositions are the same as their initial compositions and which apparently are not involved, are not, as a rule, included in the chemical equation. Solvents and their actions are frequently omitted.

These brief considerations may serve to illustrate some of the limitations of the theoretical views.

The formulations which have been developed for some apparently complex reactions, which have been shown to be made up of two or more successive simple reactions, may be given. If, in studying the kinetics of such changes, one of these simple reactions proceeds at a much slower rate than the others, this will be the reaction whose velocity is measured in place of the complete complex change. If the velocities of the separate consecutive reactions are more nearly equal, then the mathematical treatment is more complicated. For example, in the simplest case of two consecutive monomolecular reactions, $A \rightarrow B \rightarrow C$, starting with a mols of A, after a time t, x mols of A, y mols of B, and z mols of C are present. Then

$$x + y + z = a. \tag{14}$$

Also, if k_1 is the velocity constant of the reaction $A \rightarrow B$, and k_2 of the reaction $B \rightarrow C$, then

$$-\frac{dx}{dt} = k_1 x, \tag{15}$$

$$\frac{dz}{dt} = k_2 y, \tag{16}$$

and

$$\frac{dy}{dt} = -\frac{dx}{dt} - \frac{dz}{dt} = k_1 x - k_2 y. \tag{17}$$

On integration

$$a - z = a\left(\frac{k_2}{k_1 - k_2}\, e^{-k_1 t} + \frac{k_1}{k_2 - k_1}\, e^{-k_2 t}\right). \tag{18}$$

In order to use these equations certain simplifications are necessary. This has been done for a number of cases. Reference will be made again to these consecutive reactions.

The question of reactions in heterogeneous systems is of importance. As a comparatively simple case, the solution of a solid in a liquid may be considered. In reality this reaction or process consists of two consecutive reactions: (1) a reaction between solid and solvent, and (2) the diffusion away of the products of the reaction. In general terms, if the concentration of the saturated solution in immediate contact with the solid is denoted by a, the concentration of the rest of the solution at any time t by x, and s the area of the surface, then the rate of solution is given by the equation

$$\frac{dx}{dt} = ks(a - x), \tag{19}$$

and on integration

$$ks = \frac{1}{t} \log_e \frac{a}{a - x}. \tag{20}$$

The surface s is taken to be constant. This equation was found to hold for the solution of benzoic acid in water, etc.[5] With the first action extremely rapid, the velocity of the reaction is given by the rate of diffusion of the saturated solution into the rest of the liquid. This conception was then extended[6] to include all reactions occurring in heterogeneous systems, the equilibrium at the surface of two phases being set up very rapidly, especially in comparison with the velocity of diffusion. Since the equation representing the velocity of diffusion is similar in form to the equation of a monomolecular reaction, whenever the latter appears to hold for a reaction taking place in a heterogeneous system, it is probable that the reaction velocity measured is that of a rate of diffusion. Following this, it was shown[7] that in the mechanism of heterogeneous reactions, the reaction velocity is limited by the rate of diffusion of the reacting substances to the surface where the reaction is taking place through an adsorbed film of variable thickness of the substances taking part. Finally, the most recent view[8] states

[5] A. A. Noyes and W. R. Whitney, *Z. physik. Chem.*, *23*, 689 (1897).
[6] W. Nernst, *Z. physik. Chem.*, *47*, 52 (1904).
[7] C. G. Fink, *Dissertation*, Leipzig, 1907; M. Bodenstein and C. G. Fink, *Z. physik. Chem.*, *60*, 1, 46 (1907).
[8] I. Langmuir, *Jour. Amer. Chem. Soc.*, *37*, 1139 (1915).

that "In a heterogeneous chemical reaction, the activity of a surface depends in general upon the nature of the arrangement of, and spacing of the atoms forming the surface layer" and postulates that the velocity of heterogeneous reactions is controlled primarily by the rate at which the molecules strike against that portion of the surface which is active. It was recognized that physical factors such as rates of diffusion through layers of gas or through films may modify the conditions or limit the chemical reaction occurring, but these factors were considered to be of secondary importance in most cases. The velocity of a reaction then usually depends on the fraction of the surface covered by adsorbed atoms or molecules, which in turn depends on the rate of condensation and on the rate of evaporation (for gases, and corresponding rates for liquids) of the adsorbed substances. This conception was developed in mathematical form to cover certain special cases after making some simplifying assumptions. A "law of surface action" was obtained analogous to the "law of mass action." The action of a "poison" was shown to consist of the formation of a very stable film one atom or molecule deep.

These relations are of interest here because enzyme reactions frequently take place in heterogeneous systems and it is possible that some form of the theories developed will be found applicable to the kinetics of their actions. Further, enzymes as a rule, occur as colloids as will be brought out in later chapters. Just how far colloids may be considered to be in homogeneous phase in aqueous solution is an open question at present. Every case must be considered on its own merits, but the possibility of the velocity of the reaction measured not being that of the supposed chemical reaction must always be kept in mind.

The kinetic equations have been applied to chemical reactions so far in a more or less simple manner with apparently satisfactory results. However, it has been found that more careful investigations have brought to light facts which did not agree with the simple laws of kinetics to which they apparently conformed. These relations will be considered more in detail in the next chapter.

Part of the exceptions to the simple kinetic equations may be found in the use of concentrations in place of "active masses." The simple laws of chemical kinetics apply only in dilute solutions or at concentrations where the simple gas laws hold. Strictly speaking,

the concentration term holds throughout a given reaction if the relation between concentration and active mass does not change. For pure substances, the vapor pressure gives a measure of the active mass, and if the change in vapor pressure, the active mass, and the concentration run parallel, then the kinetic deductions hold. These relations apply to the equilibrium constants of chemical reactions, whether these be derived from kinetic or thermodynamic standpoints. In both cases, limitations of concentration must be introduced and the simplest criterion is that supplied by the gas laws.

It follows, therefore, that if the kinetic equations do not conform to the chemical equations, the conditions under which the kinetic equations are applied must be carefully scrutinized. There is at present no reason to assume that the law of mass action does not hold, but the method of applying it, especially in concentrated solutions, requires further study.

At the same time the possibility to which reference was made earlier in this chapter, must be kept in mind; namely, that the kinetic or mathematical equation does not necessarily represent the change taking place in the chemical equation, and that in turn the chemical equation reproduces perhaps only partially or incompletely the chemical reaction in question. These factors, as pointed out, may limit the facility with which the kinetic relationships may be used.

The brief outline of reaction velocity concepts which has been given is necessary here because enzymes are defined as substances which cause changes in the velocities of certain chemical reactions. An attempt will now be made to show the connection between these phenomena and chemical change in general. A general theory of chemical reactions will be presented, then catalytic actions as a group of chemical actions, and finally enzyme actions as a group of catalytic actions. In this general scheme enzymes find a simple and natural place. This justifies a brief presentation here. More complete details were given in another connection.[9]

The theory of chemical reactions which will be used is generally known as the addition theory.[10] According to this theory, when two

[9] K. G. Falk, "Catalytic Action," published by The Chemical Catalog Company, Inc., New York, 1922.

[10] Cf. also K. G. Falk, "Chemical Reactions; Their Theory and Mechanism," published by the D. Van Nostrand Company, New York, 1920.

or more molecules react, the first step consists of the formation of an addition compound between these molecules. This addition compound then reacts further to form the products which are finally observed.

In any given case, two or more products may react to form a complex addition compound. The latter may then in turn react further to form a variety of different products. Thus, the reaction between ammonia, hydrogen chloride, and water may be formulated as follows:

$$\left[\begin{matrix}NH_3\\HCl\\H_2O\end{matrix}\right] \begin{matrix}= NH_3 + HCl + H_2O & (a)\\ = NH_4Cl + H_2O & (b)\\ = NH_4OH + HCl & (c)\\ = NH_3 + H_3OCl & (d)\end{matrix} \quad \text{I}$$

In this formulation, the complex addition compound is indicated by the components within the brackets, but the actual linkings of this compound are not indicated. Starting with the products (a), the addition compound is formed which may then go back to (a) or to (b), (c), or (d). The reaction actually observed will depend upon the equilibrium constants of the four separate reactions if equilibrium is attained, or the concentrations of the various components and the relative reaction velocities if equilibrium is not reached. The reaction between ammonia, hydrogen chloride, and platinic chloride might be formulated similarly:

$$\left[\begin{matrix}2NH_3\\2HCl\\PtCl_4\end{matrix}\right] \begin{matrix}= 2NH_3 + 2HCl + PtCl_4 & (a)\\ = 2NH_4Cl + PtCl_4 & (b)\\ = (NH_3)_2PtCl_4 + 2HCl & (c)\\ = H_2PtCl_6 + 2NH_3 & (d)\end{matrix} \quad \text{II}$$

In this formation, the possible action of water is omitted. As written, platinic chloride takes the place of water in I. The reaction between an alcohol and an organic acid in the presence of hydrogen chloride would be formulated as follows:

$$\left[\begin{matrix}C_2H_5OH\\CH_3CO_2H\\HCl\end{matrix}\right] \begin{matrix}= C_2H_5OH + CH_3CO_2H + HCl & (a)\\ = CH_3CO_2C_2H_5 + H_2O + HCl & (b)\\ = C_2H_5Cl + CH_3CO_2H + H_2O & (c)\end{matrix} \quad \text{III}$$

A number of additional equilibria are possible in this case, but those given illustrate the method. In every case, the products actually

observed depend upon the principles outlined. In reaction III, products corresponding to equation (c) may be obtained if a tertiary alcohol is used in place of the ethyl alcohol.

The theory of chemical reactions outlined evidently introduces complications into the treatment from the standpoint of reaction velocity. It is possible that the lack of success which has attended so many of the attempts to apply the kinetic equations to chemical reactions is due to this cause. The decomposition of the addition compound in the various ways in any one case is characteristic for that compound and for the given conditions. The amounts of products obtained from this addition compound in any one equilibrium equation will naturally depend upon the concentrations of the substances which may be formed and the velocity of the reverse reaction. If the velocity of the decomposition of the addition compound alone is being measured, then the reaction will obey the monomolecular law; if the reaction is made up of two of the equilibria indicated, and if one of the reactions takes place much more rapidly than the other (the one reaction forming the addition compound, the other decomposing it), then the velocity observed will be that of the slower reaction (consecutive reactions). For reactions with inorganic compounds it is probable, in general, that with a given set of conditions and starting with certain substances, the addition compound is formed and then reacts further mainly according to one definite course. With organic compounds and materials of biological origin, on the other hand, there are, as a rule, a greater number of possibilities of reactions, and consequently a greater number of equilibria, and, unless one reaction predominates, a more complex formulation of the kinetics of the changes.

A certain awkwardness is apparent in the descriptions of the mechanisms of the reactions involving a complex intermediate compound. In the formulations of the different possibilities of a reaction, at times equilibria are spoken of without the intention of conveying the meaning that the various substances taking part are present at definite equilibrium concentrations. The term equilibrium is used in these cases in place of the more usual "chemical equation" to emphasize the significance of reversibility and mass action effect.

A catalytic reaction will be defined as a reaction in which the chemical composition of one of the initial substances is the same

as that of one of the final substances. This view was developed in some detail elsewhere.[11] A few illustrations and deductions will be given here. The product or substance whose chemical composition is unchanged as a result of the reaction in which it takes part is known as the catalyst. For example, in reaction I, equilibria (a) and (b), water would be considered to be the catalyst in the formation of ammonium chloride from ammonia and hydrogen chloride. A catalyst is generally assumed to increase the velocity of the reaction. However, in a number of cases, retardations have been observed when small amounts of certain substances were added, and the phenomena termed negative catalysis.[12] Also, a small quantity of the catalytic substance is supposed to be able to change the velocities with large amounts of the other reacting substances. Both these relations are included in the view of catalysis given here. If a reaction between two substances to form two other substances takes place at a definite rate, and the addition of a third substance changes that rate, three possibilities exist. Addition compounds may be formed made up of the two substances and of the two substances plus catalyst. The combination of catalyst and either one of the reacting substances obviously is also possible but will not be considered at this point. The formation of these two addition compounds and their decompositions evidently represent simultaneous reactions. If the velocity of the reaction involving the catalyst is less than that of the other reaction (actually the sum of the velocity of the formation and the decomposition of the addition compound in each case is meant) then probably only a small part, if any, of the reaction will follow that course and the velocity will be practically unchanged (unless the catalyst is present in great excess when the rate may be smaller). The velocities of the simultaneous reactions may also be equal. In the third case, the reaction with the catalyst is the more rapid and will be the reaction measured. This last is generally true with the substances recognized as catalysts. If a substance does not increase the velocity, it apparently does not take part in the reaction and is not called a catalyst. Negative catalysis may also be due to the course of the reaction being changed or a different set of products formed

[11] Cf. "Catalytic Action."

[12] Cf. G. Bredig, *Ergebnisse der Physiologie, 1,* 134 (1902); J. Stieglitz, *Proc. Congr. of Arts and Sciences, St. Louis, 1904, Vol. IV,* p. 276; and others.

because of the presence of the catalyst. In reactions I (a) and (b), a small amount of water would be able to take part in the reaction with considerably larger amounts of the other substances. Again, considering reactions II (a) and (b), platinic chloride catalyzes the reaction between ammonia, hydrogen chloride, and ammonium chloride; from reactions II (a) and (c), hydrogen chloride, may act as the catalyst; and from reactions II (a) and (d), ammonia may act as the catalyst. Similar reasoning may be used with reactions I. The addition of the catalyst increases the number of possible sets of equilibria. At the same time, due to the nature and properties of the catalytic substance, one of the possible reactions will be favored over the others as a rule and the reaction observed takes that course. While these relations are true, it is evident that catalytic reactions follow the same rules as chemical reactions in general, and in fact are simpler in the sense that the chemical composition of one substance is unchanged. The significant fact in the present connection is that the change in the velocity of the reaction is evidence that the catalyst substance has taken some part in the reaction.

It is hardly advisable to review here the work and conclusions of others on catalytic reactions. Reference may be made to the publications of Berzelius[13] who was the first to group reactions in a general class as catalytic; Ostwald,[14] who classified and systematized catalytic reactions; Bredig,[15] who published in 1902 a very complete review of catalytic actions and the chemical bases underlying the reactions; Mellor, who summarized in his "Chemical Statics and Dynamics" the theories developed on the subject and listed numerous references;[16] and Stieglitz,[17] who presented much valuable material and outlined important points of view on the basis of his theoretical and experimental studies on the saponification of imido esters and related compounds.

The kinetic equations which were given in the earlier part of this chapter will be taken up briefly in the present connection. The

[13] J. Berzelius, *Jahresberichte, 13,* 237 (1836); *20,* 452 (1841); *Ann. Chim. Phys. (3) 61,* 146 (1836); *Lehrbuch d. Chem. 3te Aufl. 6,* 19-25 (1837).

[14] W. Ostwald, "Ueber Katalyse," Leipzig, 1902; *Lehrb. d. Allgem. Chem. 2te Aufl.* (1903), p. 515.

[15] G. Bredig, *Ergebnisse der Physiologie, I,* 134-212 (1902).

[16] J. Mellor, "Chemical Statics and Dynamics," 1904. Cf. especially Chapter X.

[17] J. Stieglitz, *Proc. Congr. of Arts and Sciences, St. Louis, 1904, Vol. IV,* pp. 276-84; *Am. Chem. Jour., 39,* 418 (1908); *Jour. Amer. Chem. Soc., 32,* 225 (1910).

constant k of the reaction velocity equations is a constant characteristic for the chemical reaction in question under certain conditions. In every case k represents the amount of change in unit time starting with unit concentration and keeping the concentration constant throughout the unit of time. As stated before, the effect of temperature shows itself in a two- to three-fold increase in k for an increase of 10 degrees. The increase in the rate of diffusion for a similar rise in temperature is very much smaller, so that this has been used to determine whether certain measured velocities were due to chemical reaction or to diffusion.

Now, k is assumed to be constant if conditions do not change. It may be asked what is meant by conditions which do not change. The action of a catalyst may change the velocity of a reaction and therefore the value of k even when the catalyst substance is apparently unchanged by the reaction. In a reaction taking place in a gaseous system, it has been found that the presence of an indifferent gas does not change the value of k, the volume being kept constant. In a solution, however, matters are different. If a reaction is studied in a number of different solvents, it is found that the velocity at a definite temperature and concentration may vary widely. The example which is quoted most frequently in this connection is that studied by Menschutkin.[18] He investigated in a large number of organic solvents the reaction between triethylamine and ethyl iodide to form tetraethylammonium iodide. The reaction in every case followed the equation for the bimolecular law, but the values of the velocity constant k were different with different solvents, ranging from 0.000180 to 0.133 for the conditions used. The obvious explanation of these results is that the solvent takes part in the reaction in some way. The replacement of part of a solvent by another, such as water by alcohol, may change the velocities of the reactions taking place in them considerably. Reference may be made to the careful studies on the hydrolysis of esters under such conditions by McCombie and his associates.[19]

The velocity of a reaction, or the value of k, may then be changed

[18] N. Menschutkin, *Z. physik. Chem.*, *6*, 41 (1890).

[19] A. E. Cashmore, H. McCombie and H. A. Scarborough, *J. Chem. Soc.*, *119*, 970 (1921); *121*, 243 (1922); *123*, 197 (1923); H. McCombie, H. A. Scarborough, and R. H. Settle, *J. Chem. Soc.*, *121*, 2308 (1922); J. Dexter, H. McCombie and H. A. Scarborough, *J. Chem. Soc.*, *123*, 1229 (1923); W. J. Jones, H. McCombie and H. A. Scarborough, *J. Chem. Soc.*, *123*, 2688 (1923).

by various amounts of added substances, ranging from a change in solvent to the addition of minute quantities of catalysts, such as acid in sucrose hydrolysis, etc. The latter have always been included under catalysts, and it is a question as to how far the term catalyst should go. Catalysts are often defined as substances present in small amounts and having the properties indicated. The definition of amount offers a real difficulty, however. There seems to be no reason to limit the definition of catalyst in this way. If the velocity of the reaction is changed by the change in solvent, it appears as though the solvent takes an active part in some way in the reaction. From the theory used here, it would be involved directly in the formation of the addition compound. In other cases, more direct evidence of such addition compound formation is available. In other words, a substance may be present in any quantity and may be considered to act as a catalyst.

To sum up some of these relations, chemical reactions between two or more substances take place with the primary formation of addition compounds which then react further. The velocity observed in any given case depends upon the velocities of the separate reactions. In a catalytic action, the chemical composition of one of the reacting substances or components, perhaps in the sense that component is used in phase rule discussions, is the same before and after the reaction, but the velocity of the reaction observed is different. Not every substance which is unchanged in composition but forms addition compounds and takes part in a reaction need change the observed velocity.

If k is the velocity constant for a given reaction and k_1 the velocity constant in the presence of a catalyst, then the comparative values of k_1 and k show the relative speeds in the two cases. If it is desired to compare the rates of the reaction under the two conditions, since the concentration of the catalyst may be assumed to be constant especially during the first part of the reaction, then the ratio of the times for the same amount of the chemical change in the two cases will be inversely proportional to the values of the constants. That is,

$$\frac{t_1}{t} = \frac{k}{k_1}. \qquad (21)$$

In this way, a correct measure of the rates of the reaction in the presence and absence of the catalyst will be obtained. To determine the chemical changes in the same period of time in the two cases will usually give a close indication of the relative rates, but may, at times, lead to quite erroneous conclusions.

The increase in the value of k which has been shown to be characteristic of a catalytic action, even if not the fundamental criterion of such action, can take place in two ways. In the first place, a definite increase in k may be observed as due to the presence of a definite amount of catalyst. The value of k is constant for the new reaction, but greater in magnitude (smaller for negative catalysis). Again, the value of k may increase continuously throughout the reaction due to the formation in the course of the reaction of new catalyst substance, in addition to the catalyst substance present initially. For example, the hydrolysis of an ester by an organic acid would be a case in point, the formation of ethyl acetoacetate from ethyl acetate, sodium, and a minute quantity of alcohol, etc. The first type would represent a definite increase in velocity, the second type would represent accelerated reactions. These last have frequently been termed autocatalytic.

The presence of a catalyst is not supposed to change the equilibrium of a chemical reaction according to the views used heretofore in most discussions. However, a number of workers have expressed themselves more definitely at various times showing the conditions which limited this view.[20]

A general conclusion may be stated and reference made to papers published elsewhere for a more complete discussion. Unless the catalyst is entirely uncombined, or is combined in the same way before and after the reaction has taken place, the equilibrium constant of the reaction may not be the same in the presence and absence of the catalyst. That is to say, if work is done in introducing into or removing the catalyst from the reaction mixture, some sort of a chemical compound is formed between two or more molecular species, whether this be called chemical combination, solution, adsorption, physical change, etc., and the equilibrium may

[20] Cf. G. Bredig, *Ergebnisse der Physiologie, 1,* 139 (1902); E. Abel, *Z. Elektrochem., 13,* 555 (1907); J. Stieglitz, *Amer. Chem. Jour., 39,* 56 (1908); M. A. Rosanoff, *Jour. Amer. Chem. Soc., 35,* 173 (1913); W. D. Bancroft, *Jour. Physic. Chem., 21,* 573 (1917); K. G. Falk, "Catalytic Action," 1922, Chapter IV; and others.

be changed. In the ideal limiting case of no combination or combination to the same extent, the equilibrium of the reaction will not be changed by the presence of the catalyst substance.

The application of these considerations to the velocities of catalytic reactions is simple in principle. As stated earlier, the equilibrium constant is taken to be made up of the two velocity constants of the opposing reactions. If the equilibrium of a reaction is unchanged by the addition of a catalyst, and if the catalyst increases the velocity of the reaction in one direction, then it must, to a corresponding degree, act as a catalyst for the opposing reaction and increase its velocity. This deduction was made a number of years ago, and the general qualitative statement has been found to be true, although quantitative results to test this point are not at hand in sufficient amount for satisfactory conclusions to be reached. If, however, the equilibrium of a reaction is changed by the presence of a catalyst, then the velocities of the opposing reactions need not be affected by the catalyst to the same extent, and it is readily conceivable that the velocity of a reaction in one direction might be increased, while that of the reaction in the opposite direction not be affected at all. This may be tantamount to saying that the catalyst for the one reaction did not act as a catalyst for the opposing reaction, and from the preceding discussion might be explained chemically by considering that the catalyst substance was combined with some substance in the reaction mixture which prevented it from taking part in the desired reaction.

On the other hand, if by definition, a catalyst cannot change the equilibrium of a reaction, then a discussion of the question is unnecessary.

Enzyme actions may be considered to be a group of catalytic actions in which the catalyst, the enzyme, is derived from biological sources. Possibly because of the nature of their sources, they show certain properties, both chemical and physical, which serve to characterize them and which will be described in later chapters. In general, however, the main features of enzyme actions may be grouped and classified as catalytic actions, the same general principles being applicable to all.

III.—Chemical Reactions Catalyzed by Enzymes

The chemical reactions whose velocities are increased by enzyme preparations include a number of comparatively simple reactions as well as many complex ones. That is to say, the transformations which take place may involve simple chemical changes which are more or less well known and which can be followed satisfactorily. It is with these reactions that it would appear that further insight into the enzyme problem will be gained. It is true that many things can be done and advances made even by means of enzymic reactions with such complex substances as proteins and starches. In these, however, the twofold difficulty of dealing on the one hand with substances which, while chemically characterized as belonging to certain types, are unknown even so far as their chemical composition is concerned, and on the other hand using to change the velocities of reactions, preparations which are chemically practically entirely uncharacterized, makes it appear as if more is to be hoped for at the present time with the simpler reactions.

In this chapter, some of the reactions which are to be included in enzyme actions will be considered entirely aside from the enzyme part of the problem. It is not necessary to enter into all of the reactions, even of the simpler ones, which might be included. Of the hydrolytic reactions, the hydrolysis of sucrose and of esters will be considered somewhat in detail. Then some of the changes which urea and some of the simpler carbohydrates may undergo will be described as well as some observations on the hydrolysis of gelatin, and finally, oxidation reactions will be taken up briefly.

The two hydrolysis reactions first mentioned were chosen because they have been studied very extensively from the chemical point of view, and because the enzyme preparations which influence the velocities of the reactions have also been studied to considerable extents. It should be obvious that, with the enzyme as the unknown factor, as much information as possible with regard to the chemical reaction itself should be at hand in order to elucidate the

action and nature of the unknown factor, the enzyme. In the rest of this chapter, enzymes will be considered only secondarily. Attention will be focused on the chemical reaction as such.

The empirical equation representing the hydrolysis of sucrose may be written as follows:

$$\underset{\text{sucrose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{fructose}}{C_6H_{12}O_6} \qquad (1)$$

The possible action of other substances present in solution is not given. This equation represents, however, only the barest outline of the changes occurring. Questions of isomerism must also be considered. A more complete statement of the changes taking place may be shown in the following outline:

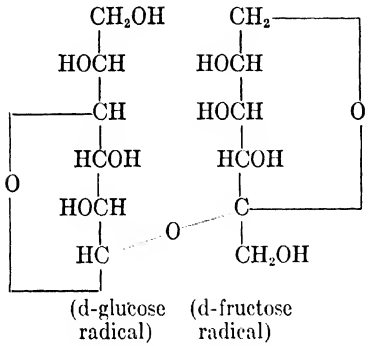
$$\text{Sucrose} + \text{Water} \begin{cases} \nearrow \alpha\text{-glucose} + \beta\text{-glucose} \\ \searrow \alpha\text{-fructose} + \beta\text{-fructose} \end{cases} \qquad (2)$$

Equations (2) may be written in such a way as to indicate the structural formulas of the various substances involved. This is done in equations (3) on page 51. Although these formulas cannot be considered in any sense final, they will serve to indicate the complexity of the conditions which may be met in the study of this reaction.

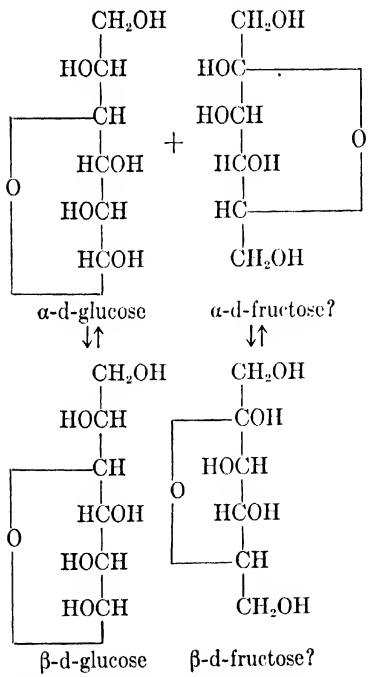
α-Fructose has not been isolated as yet in the pure state. The structural formulas to be assigned to the two fructoses are not quite definite, but it seems to be very probable that there is an α- and β-form, and that an equilibrium exists in solution between these forms.

Sucrose is in all probability a compound consisting of a butylene oxide aldose (d-glucose radical) and an amylene oxide ketose (d-fructose radical). It is not a simple glucoside or fructoside. In the hydrolysis, α-glucose is formed which is then partially converted into β-glucose until an equilibrium mixture is present (butylene oxide forms). In the hydrolysis reaction, the fructose portion of the original sucrose molecule first forms an unstable, reactive fructose (γ-fructose) which rapidly goes over into the ordinary α- and β-fructose forms (butylene oxide structure).[1] The

[1] J. C. Irvine, *J. Chem. Soc.*, *123*, 898 (1923); *Ind. Eng. Chem.*, *15*, 1162 (1923).



$+ H_2O =$



(3)

gradual development of the knowledge of γ-sugars has given a new impetus to the study of the reactions of the simple hexoses. Structurally, the name γ-sugar is applied "to all forms of sugars in which

the oxygen ring is displaced from the normal, stable position." No simple sugar has definitely been isolated in the γ-form, but a number of their methyl derivatives have been studied. They are characterized by their instability or reactivity. For example, they undergo oxidation with extreme ease, trimethyl-γ-glucose reducing Fehling solution instantaneously at room temperature; they are all convertible into glucosides with remarkable readiness, etc. Their reactions show close resemblances to those observed in natural processes. Undoubtedly, their further study will throw much light on such processes and it is to be hoped that their structural relationships to the stable sugars isolated in the laboratory will soon be elucidated.

To return to the hydrolysis of sucrose and the present status of the knowledge concerning the reaction, the probable primary or intermediate compound between sucrose and water which precedes the formation of the hexoses is not indicated in the formulations. The first step mentioned is the reaction between sucrose and water to form α-glucose and an unstable form of fructose. This reaction, taking place as a rule in aqueous solution, is, as far as known, irreversible under the conditions which have been used. The α-form of glucose reacts further going over into the β-form, while the unstable form (γ) of fructose goes over into probably a mixture of the ordinary levorotatory α- and β-forms of fructose. The mechanism of the change from the α- to the β-form, known as mutarotation, or vice versa, in all probability depends upon the formation with water of an intermediate compound, probably a monohydrate.[2]

As stated earlier in this chapter, it is advisable to know as much as possible of the chemical and physical properties of the substances whose chemical changes are being followed kinetically. Before going on to the velocity of the indicated reaction, a few of the properties of the substances may be referred to. The rotation of the plane of polarized light by these compounds in solution is perhaps the property of which use is most commonly made. The spatial arrangements of the atoms and groups is indicated in the formulas of equations (3). The change in rotation in the various

[2] T. M. Lowry, *J. Chem. Soc.*, 75, 213 (1899); 83, 1314 (1903); 85, 1551 (1904). E. F. Armstrong, *J. Chem. Soc.*, 83, 1305 (1903). J. W. Baker, C. K. Ingold, and J. F. Thorpe (*J. Chem. Soc.*, 125, 268 (1924), however, consider mutarotation of sugars to be a case of ring-chain tautomerism and not dependent on the intermediate formation of hydrates.

stages of the reaction are marked enough to permit of the determinations of the chemical transformations by these means. The specific rotation of sucrose is $[\alpha]_D^{20^\circ} = +66.7$.[3] For α-d-glucose the best value was found to be 111.2°, for β-d-glucose 17.5°. In aqueous solution at equilibrium the value for a mixture of α- and β-glucose is 52.5°; or the amount of α-form present is 37.4% and of β-form 62.6%. This equilibrium does not change appreciably with the temperature. The temperature coefficient of the specific rotation of fructose is a function of the concentration, as well as of the temperature. At equilibrium at 25° C., the value of the rotation is −88°. The specific rotation for β-fructose was found to be −130.8° between 0.15° and 37°. Since α-fructose is not known in a pure state, its value could not be determined directly, but it has been estimated by indirect methods to be +17°. Increase in temperature changes the equilibrium in favor of the α-form.[4]. At 87° C. the mixture shows a rotation of −52.5°. A mixture of equal parts of d-glucose and d-fructose at this temperature will therefore show zero rotation, since the temperature coefficient of the rotation of glucose is practically zero.

Another property which may be of importance in some cases in following these changes in solution, is the viscosity of the solution. For solutions which are concentrated to any considerable degree, since kinetic actions are involved, it would appear to be advisable to refer all changes to solutions possessing the same fluidity or viscosity.

As for the chemical properties or reactions, the one which is used frequently is the reducing action of the hexoses formed, on alkaline cupric salt solutions such as Fehling solution and others. Sucrose does not react under certain definite conditions, so that the amount of change can be followed by the amount of cuprous oxide formed. The hexoses are oxidized in the reaction, and it is obvious that it is necessary to adhere strictly to fixed conditions which have been standardized with known solutions to obtain comparable results, because of the manifold possibilities of oxidation of the hexoses.

[3] Cf. J. E. Mackenzie, "The Sugars and Their Simple Derivatives," 1914, pp. 28-9.

[4] J. M. Nelson, and F. M. Beegle, *Jour. Amer. Chem. Soc.*, *41*, 559 (1919). Cf. also C. S. Hudson, *Ibid.* *31*, 655 (1909); C. S. Hudson and E. Yanovsky, *Ibid.* *39*, 1013 (1917); W. C. Vosburgh, *Ibid.* *42*, 1696 (1920).

If this is done, accurate results may be obtained,[5] and in certain cases this method possesses advantages over other methods of following the changes. The property of the reducing actions of the hexoses may be used with other reagents [6] but the general principles of the reactions remain the same.

The hydrolysis of sucrose has been assumed so far to take place in aqueous solution without the addition of any other substance. At moderate temperatures this reaction is extremely slow. Acids increase the velocity of the reaction, and, as a first approximation, with monobasic acids, the greater the strength of the acid as measured by its electrolytic dissociation or ionization in solution, the greater its catalytic action. The velocity of the reaction has been measured a number of times, and the kinetic equations outlined in the last chapter applied. Their application appears simple at first sight, but closer study has revealed a number of complicating factors. In solutions so dilute that the active mass (or concentration) of the solvent does not change appreciably during the measurements, the velocity of the reaction might be expected to follow the monomolecular law, that is, the amount of change being proportional to the concentration of sucrose present at any instant. The two methods of measuring the change which have been used are the change in rotation in the plane of polarized light, and the reduction of alkaline copper salt solution. The reaction in which the α-forms of the hexoses are transformed to the β-forms is apparently involved in the measurements, but it was found that equilibrium between the different forms is reached fairly rapidly in the presence of acids.

The proportionality between the hydrogen ion concentration and the velocity of the reaction was found to hold quite satisfactorily in dilute solutions of acids as stated in the last chapter. However, in the more concentrated solutions of the acids, or in the presence of added salts, this proportionality no longer was found to hold. That is to say, the values of k of the velocity equations were no longer

[5] Cf. H. C. Sherman, E. C. Kendall and E. D. Clark, *Jour. Amer. Chem. Soc.*, **32**, 1073 (1910), for comparison of different methods of determining reducing actions of sugars; also F. A. Quisumbing and A. W. Thomas, *Jour. Amer. Chem. Soc.*, **43**, 2713 (1921).

[6] Such as picrate. Cf. R. C. Lewis and S. R. Benedict, *Proc. Soc. Exp. Biol. and Med.*, **11**, 57 (1913-4); *J. Biol. Chem.*, **20**, 61 (1915); S. R. Benedict and E. Osterberg, *J. Biol. Chem.*, **34**, 195 (1918); R. Okey, *J. Biol. Chem.*, **38**, 33 (1919); W. M. Dehn and F. A. Hartman, *Jour. Amer. Chem. Soc.*, **36**, 403 (1914); K. G. Falk and H. M. Noyes, *J. Biol. Chem.*, **42**, 109 (1920).

constant for a series of determinations with the more concentrated acid, or the ratio of the constant of the velocity equation to the hydrogen ion concentration varied with different concentrations of the acid. In order to account for these variations, modifications of the original theory were proposed. A brief review of some of the various theories suggested may be of interest in this connection.

In considering the catalytic actions of acids on the hydrolysis of sucrose, three general sets of explanations or theories to account for the reactions have been proposed and are used at the present time. In the first place, the action has been considered as due entirely to the hydrogen ions present in solution; second, the so-called Dual Theory of Catalysis assumes the action to be due both to the hydrogen ions and to the unionized molecules; third, the addition theory of chemical reactions assumes the intermediate or primary formation of an addition compound with the acid molecule or possibly one of the ions, and considers that the action of the solvent is involved as one of the main factors, while the ionization is secondary.

The three points of view will now be considered in more detail since they apply to a great many reactions, especially to those under consideration. In the first, the action is stated to be due entirely to the hydrogen ion. The velocity constants of the sucrose hydrolysis with acids were found to be practically proportional to the equivalent conductivity of the acid in dilute solutions. Since this equivalent conductivity is due mainly to the concentration of the hydrogen ion, the catalytic action was taken to be due to the hydrogen ion. Other reactions whose velocities were increased by acids were found also to parallel very closely these changes and were used as additional evidence. This relation was developed between 1880 and 1890 and received strong support from the theory of electrolytic dissociation of Arrhenius.

As data on this reaction accumulated, especially with more concentrated solutions, the simple explanation of the action of hydrogen ions was found to be insufficient. For example, nitric acid, 0.5 N, containing according to conductance determinations 4.64 times as many hydrogen ions as a 0.1 N solution, hydrolyzed a sucrose solution 6.07 times as fast.[7] While this and similar apparently "abnormal" actions of acids may be explained away in various ways,

[7] Cf. J. W. Mellor, "Chemical Statics and Dynamics," 1909, p. 280.

greater difficulties are encountered in the reactions where neutral salts are added, which, in place of decreasing the actions because of the repression of the ionization of the acid, frequently increased them. These phenomena were developed for a number of reactions especially from 1907 on by S. F. Acree, G. Senter, S. A. Arrhenius, G. Bredig and H. C. Snethlage, H. Goldschmidt, A. Lapworth, H. S. Taylor, H. M. Dawson, and others.[8] The explanation advanced and called by Dawson the Dual Theory of Catalysis, takes the view that in addition to the catalytic action of the hydrogen ion, the unionized acid molecule exerts a catalytic action. For a number of reactions and with a number of acids the catalytic action of the unionized acid molecule, denoted by k_m, and the catalytic action of the hydrogen ion, denoted by k_h, were determined, using the gram-equivalent as the unit of mass.[9] The ratio of these values gives the comparative effect of the catalytic actions of acid molecule and hydrogen ion. The strongest acids as measured by the ionization gave the largest values for the ratios k_m/k_h, while the weak acids gave the smallest values. The values of the ratios ranged from about 2 for acids such as hydrochloric acid to less than 0.01 for very weak acids. This ratio, assumed at first to be characteristic for a given acid, was found later to vary for any one acid with the reaction being catalyzed. If intermediate compounds with the catalyst are formed, a reason for this variation is apparent.

The Dual Theory of Catalysis and the catalytic actions of unionized molecules leads to the third theory of the actions. The presence of hydrogen ions, or the phenomenon of electrolytic dissociation, is ascribed to certain properties of the solvent as in all the theoretical views. In the reaction catalyzed by the acid, an addition compound is assumed to be formed by the acid, sucrose, and water of the solvent, this primary addition compound then being able to react in several different ways, for instance, to form sucrose and

[8] Cf. the summary in W. C. McC. Lewis, "A System of Physical Chemistry," 1918, Part I, pp. 423-9. The historical development of the views is of interest, but can only be referred to here. For example, the first suggestion of the possible activity of unionized molecules in catalytic actions was made by H. Goldschmidt in 1899 (*Z. physik. Chem. 29*, 118); the increasing importance ascribed to the simultaneous actions of unionized molecules and of hydrogen ions by J. Stieglitz (*Am. Chem. Jour., 39*, 167 (1908)), S. F. Acree and J. M. Johnson (*Am. Chem. Jour., 37*, 410 (1907), *38*, 329 (1908)), and so on.

[9] A summary of these values was given by H. S. Taylor, *Z. Elektrochem., 20*, 202 (1914).

acid; glucose, fructose, and acid, etc., as outlined in principle in Chapter II in discussing the general theory of chemical reactions. The composition of the intermediate compound may be indicated qualitatively; quantitatively it is undetermined.[10] The question of hydrogen ions in this viewpoint naturally arises. This is not considered to be the predominating factor. The complex addition compound may ionize so that apparently it is an addition compound with the hydrogen ion, not with the acid. The ionization is considered as a secondary reaction which in itself plays no direct part but is only a physical indication that the solvent has brought about a change in the acid molecule, just as the chemical action of the sucrose hydrolysis is similar evidence of a parallel nature of the chemical change in the acid molecule brought about by the solvent.

In the discussion of the first two viewpoints and the probable action of the hydrogen ions, no allusion was made to the methods which are used to determine their concentration, or in other words, the degree of ionization of an electrolyte. Without entering into the question of the determinations of hydrogen ion concentrations, or the theoretical significance of the experimental results which have been obtained, it may be noted that the more recent work on the theory of electrolytic dissociation has tended to modify profoundly some of the basic concepts of this theory.

A number of the most active workers in this field[11] have advocated the view that for highly ionized or strong electrolytes, electrolytic dissociation is complete, and that the observed and calculated

[10] Cf., however, G. Scatchard, *Jour. Amer. Chem. Soc.*, *43*, 2387 (1921), for definite experimental evidence on this question.

[11] W. Sutherland, *Phil. Mag.* (6) *14*, 3 (1907); S. R. Milner, *Phil. Mag.* (6) *23*, 551 (1912); *25*, 742 (1913); *35*, 214, 354 (1918); J. C. Ghosh, *J. Chem. Soc.*, *113*, 449, 627 (1918); N. Bjerrum, *Z. Elektrochem.*, *24*, 231 (1918); A. A. Noyes and D. MacInnes, *Proc. Nat. Acad. Sci.*, *6*, 18 (1920), *Jour. Amer. Chem. Soc.*, *42*, 239 (1920); J. C. Ghosh, *Z. physik. Chem.* *98*, 211 (1921); G. Akerlöf, *Z. physik. Chem.*, *98*, 260 (1921); H. Kallman, *Z. physik. Chem.*, *98*, 433 (1921); R. H. Clark, *Jour. Amer. Chem. Soc.*, *43*, 1759 (1921); L. Ebert, *Jahrb. Radioaktiv. Elektronik*, *18*, 134 (1921); A. A. Noyes and M. S. Sherrill, "Chemical Principles," published by The MacMillan Company, 1922, especially pp. 123-6; H. J. S. Sand, *Phil. Mag.* (6), *45*, 129, 281 (1923); P. Debye and E. Hückel, *Physikal. Z.*, *24*, 185, 305 (1923); P. Debye, *Physikal. Z.*, *25*, 97 (1924); J. N. Brönsted, *Jour. Amer. Chem. Soc.* *42*, 761 (1920); *44*, 872, 938 (1922); *45*, 2904 (1923); J. N. Brönsted and V. K. LaMer, *Jour. Amer. Chem. Soc.*, *46*, 555 (1924); and especially A. A. Noyes, *Jour. Amer. Chem. Soc.*, *46*, 1080, 1098 (1924). Cf., however, S. K. Dunn and E. K. Rideal. *J. Chem. Soc.*, *125*, 676 (1924).

values obtained for the percentages of ionization show deviations from the values for complete ionization because of secondary relations.

Another method of treating the experimental results is that introduced by G. N. Lewis and involving a different concept, that of "activity." The significance of this term may be indicated by the following quotation: [12] "The mass-action law is a limiting law rigorously exact only for perfect gases or perfect solutes, but holding true with reasonable accuracy in the case of most gases at moderate pressures (such as 1 to 5 atmospheres), and in the case of solutes with electrically neutral molecules up to moderate concentrations (such as 1 molal), but showing large deviations in the case of ions even at small concentrations (such as 0.1 molal). For the mass-action and thermodynamic treatment of solutes at concentrations larger than those at which they can be regarded as perfect solutes, it has been found convenient to introduce a new concept, which will now be described.

"The mass-action of a perfect gas or perfect solute, as the mass-action law states, is represented by its concentration; but when a chemical substance is not a perfect gas or solute or when its concentration is unknown, its mass-action is expressed by the term *activity* (*a*), by which is meant that quantity which, when substituted for the concentration of the substance in mass-action equations, expresses its effect in determining the equilibrium. Hence the activity of a substance represents its effective concentration from a mass-action viewpoint; and the factor by which the actual concentration *c* must be multiplied to give the activity is called the *activity-coefficient* α, that is, $a = \alpha c$, or $\alpha = a/c$." The activity, or relative activity, therefore refers to a substance or state in comparison with another substance or state which is taken as the standard. The various applications of this concept have been given by Lewis and Randall.[13] It is probable that its further development will give new and useful results in connection, not only with the reactions involved with enzymes, but also with the nature of the enzyme actions themselves.

[12] A. A. Noyes and M. S. Sherrill, "Chemical Principles," pp. 169-70.

[13] G. N. Lewis and M. Randall, "Thermodynamics and the Free Energy of Chemical Substances," published by McGraw-Hill Book Company, Inc., New York, 1923.

Reference may be made to several investigations[14] in which the activity view was used in interpreting the results obtained in the hydrolysis of sucrose by acids and interesting and important conclusions developed.

The general acceptance even in part of the views indicated would necessitate the modification of the first two theories of chemical reactions outlined here, since the chemical reactions in themselves could not be used as evidence in connection with or in paralleling the degrees of ionization without further assumptions.

The third view presented above cannot at present be formulated as precisely as the two earlier theories, partly because of the more complex nature of the reactions involving the intermediate compound. It appears, however, to be more flexible and of more general applicability.

Which of these views will be accepted ultimately cannot be foretold at present. They are presented in order to show the status of the theoretical side of the problem of the catalysis of sucrose by acids. Since this monograph does not include a general treatment of catalytic reactions, the detailed evidence which has been accumulated will not be presented, nor will a critical summary of the experimental work bearing on the question be attempted. For this work the reader is referred to textbooks and other suitable summaries of the work on sugars and the studies on catalysis.

For the objects in view here, it will only be necessary to emphasize a few additional points in connection with the catalytic hydrolysis of sucrose. Increase in concentration of acid, whether measured by the increase in hydrogen ion concentration stated as values of pH,[15] or as concentration of acid equivalents or mols, or in any other way, increases the rate of hydrolysis, not quantitatively, except possibly for the very dilute solutions, but in a very rough way, proportionately, or perhaps better, in the same direction. In alkaline solution, or even neutral, the velocity of the reaction is almost nil. Increase in temperature increases the velocity two- or three-fold for every 10° rise, as with chemical reactions in general.

[14] C. M. Jones and W. C. McC. Lewis, *J. Chem. Soc., 117*, 1120 (1920); G. Scatchard, *Jour. Amer. Chem. Soc., 43*, 2387 (1921), *45*, 1580 (1923); T. Moran and W. C. McC. Lewis, *J. Chem. Soc., 121*, 1613 (1922); W. C. McC. Lewis, D. E. Merriman, and T. Moran, *Jour. Amer. Chem. Soc., 45*, 702 (1923).

[15] pH represents the negative exponent of 10 (or the negative value of the logarithm to the base 10) of the number representing the hydrogen ion concentration.

The addition of neutral salts to an acid solution increases the rate of reaction, although if acid is not present, the reaction does not take place with neutral salts alone in solution.

The reactions involving the hydrolysis of esters follow in principle closely those involving the hydrolysis of sucrose. Some additional facts have been observed which complicate the reactions in some ways and help to explain them in other ways. In the first place, in the hydrolysis of esters, the reaction is catalyzed by bases as well as by acids. Assuming the hydrogen and hydroxyl ions to be the active catalysts, it was found that for equivalent concentrations, the hydroxyl ion exerted about 1400 times as much action as the hydrogen ion. It is evident, consequently, that in passing from an acid to an alkaline solution, a hydrogen ion concentration where the acid concentration is approximately 10^{-6} normal will be found for the solution where the catalytic action of hydrolysis (or saponification) of the ester is a minimum, increasing continuously from this point with increasing concentration of acid or of alkali. This was observed by Wÿs[16] a number of years ago. In the study of the velocity of the reaction of ester hydrolysis, the formation of acid from the ester complicates the application of the velocity equations especially where alkali is used as the catalyst. Where acid is used, the reaction would be accelerated, or be autocatalytic.

The same general relations were found with the ester hydrolysis reactions as with the sucrose hydrolysis reaction. With acids, for dilute solutions, the velocity was found in general terms to be directly proportional to the hydrogen ion concentration. (For bases, similarly, proportionality was found to the hydroxyl ion concentration as illustrated in Chapter II.) For more concentrated solutions, the proportionality did not hold. Also, with a weak, or slightly ionized acid, addition of a soluble neutral salt of the acid diminished the ionization of the acid and decreased the catalytic action of the acid proportionately; but if the acid was highly ionized, addition of the salt not only did not decrease the catalytic action of hydrolysis, but actually increased it.

The different explanations or theories proposed to account for these catalytic actions are exactly the same as those given in connection with the sucrose hydrolysis. The observation of a minimum action at a certain hydrogen ion concentration is explainable by

[16] J. J. A. Wÿs, *Z. physik. Chem.*, *11*, 492 (1893).

any of the theories since ionization is not necessarily included in the explanations. Neutral salts influence the velocity of the reaction, and in the Dual Theory of Catalysis, values similar to those already indicated have been calculated for the relative actions of unionized acid molecules and hydrogen ions. These differed in some cases quite considerably from the values found for the same acids with the sucrose hydrolysis catalyses. The activity concept has also been used with the ester hydrolysis reaction and has proven useful in correlating the relations found with those obtained with other reactions.

An extended experimental and theoretical study of Stieglitz[17] must be mentioned in this connection. His general point of view was stated to be "The one vital fact, then, of an acceleration due to an increase in the active mass or concentration of a reacting component in a catalytic action is the only fundamental fact common to all catalytic actions." The mechanisms of a number of hydrolytic and similar reactions whose rates of change were increased by the addition of acids were studied and shown to be fundamentally similar. Hydrolysis of esters and the reverse reactions of esterification, for example, in which the velocities of the actions are increased by acids, involved the formation of complex oxonium ions (ester or acid plus hydrogen ion of catalyst) as the chief reacting components. The increases in concentrations of these positive ions because of the addition of highly ionized acid increased the rates of reaction. It will not be possible to enter further into these studies here or to describe the various reactions which were shown to be fundamentally related. The significance and importance of these results can only be indicated, including as they do a combination of the study of the kinetic and structural relationships of the compounds in question to elucidate the detailed mechanisms of the reactions.

As for the third explanation advanced above, experimental evidence has been obtained showing the existence of ternary compounds of acid, alcohol, and catalyzing acid, or of binary compounds of ester and catalyzing acid. This question was gone into in some detail in other connections and reference will only be made here to these publications.[18] It need only be added that questions of

[17] The work was summarized in two papers: J. Stieglitz, *Jour. Amer. Chem. Soc.*, *32*, 221 (1910); *35*, 1774, (1913); and briefly outlined in K. G. Falk, "Catalytic Action," pp. 32-35.

[18] Cf. K. G. Falk and J. M. Nelson, *Jour. Amer. Chem. Soc.* *37*, 1732 (1915),

electrolytic dissociation do not enter here any more than in the sucrose hydrolysis catalysis as a primary factor; that the original acids (or bases) may ionize, that the (intermediate) addition compound may ionize, but that these ionization phenomena are secondary (and perhaps incidental) to the reaction taking place. As stated frequently, they may be taken as physical evidence of the reacting medium or complex, being, so to speak, "active" or doing something, just as the chemical change is chemical evidence of the same thing.

The two reactions outlined indicate the present status of the views held with regard to the action of catalysts. These reactions have been studied extensively and from various points of view. They are relatively simple as far as the chemical natures of the changes are concerned. There is not, at the same time, general agreement on the theoretical views of the mechanism of these reactions. It may seem, therefore, unwarranted to consider other chemical reactions which are not as simple and which have not been studied as extensively. Most chemical changes involved in enzyme actions deal, however, with more complex materials. It is advisable, therefore, to speak of some of these reactions, although not in such detail.

The hydrolysis reactions of proteins belong to the most important types of enzyme actions. In the absence of enzymes, quantitative results on the hydrolysis of proteins by acids and alkalies are rather scanty. A recent study [19] of this question, has, however, supplied some of the desired data. Northrop followed the hydrolysis of gelatin at hydrogen ion concentrations between $10^{-0.5}$ N and 10^{-14} N at 25° and 40°. In strongly acid solution, $[H^+] = 10^{-2.0}$ N, the velocity of hydrolysis was proportional to to the hydrogen ion concentration as determined by the hydrogen electrode. In strongly alkaline solution, $[H^+] = 10^{-10.0}$ N, the rate was proportional to the hydroxyl ion concentration determined similarly. The hydroxyl ions hydrolyzed the gelatin 30 times as rapidly as did the hydrogen ions at the same concentration. This should lead to a minimum rate of hydrolysis at about $[H^+] = 10^{-6.0}$ N. Experimentally it

for references to the experimental evidence regarding the existence of these compounds; also G. Baume and G.-P. Pamfil, *J. chim. phys.*, *12*, 260 (1914); J. Kendall and co-workers, *Jour. Amer. Chem. Soc.*, *36*, 1222, 1722, 2498 (1914); *37*, 149 (1915); *43*, 1426 (1921).

[19] J. H. Northrop, *J. Gen. Physiol.*, *3*, 715 (1921).

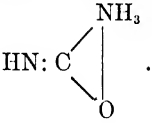
was found that a minimum rate of hydrolysis occurred at this hydrogen ion concentration, but that the actual amount of hydrolysis was 300 times as great as that calculated on the assumption that the hydrogen and hydroxyl ions acted to the same proportionate extent as in the more acid and more alkaline solutions. Since there is no reason to assume that the hydrogen and hydroxyl ions have different properties or behave differently in the neighborhood of the neutral point than in the strongly acid or alkaline solutions, the explanation was advanced that a change in the gelatin occurred at the different ranges of hydrogen ion concentrations. A comparison of the hydrolysis rate from that viewpoint which assumes proportionality of hydrogen and hydroxyl ion actions throughout, with the per cent of uncombined gelatin present at the given hydrogen ion concentrations as shown by the titration curve for gelatin, led to the conclusion that the uncombined gelatin was hydrolyzed about 200 times as rapidly as the combined (or ionized), if the efficiency of the actions of the hydrogen and hydroxyl ions was the same both for the uncombined and combined gelatin. It was found that the hydrolysis of gelatin at constant hydrogen ion concentration obeyed the monomolecular reaction velocity law for about one-third of the reaction.

These results apply directly only to the one protein studied. They are suggestive, however, and indicate that further studies, especially with simpler bodies such as peptides, will give results of interest and value. A reaction, which may be considered related in a sense, and which has been studied quite extensively, both in the absence and presence of enzymes, is the hydrolysis of urea. This reaction is formulated frequently in the following simple way:

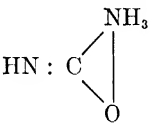
$$(NH_2)_2CO + H_2O = 2NH_3 + CO_2. \qquad (4)$$

This simple carbamide formula for urea has been generally taken to be satisfactory and to account for most of the properties and reactions of the compound. At the same time the fact that these views are not, in any sense, final, must be pointed out. In the first place, certain reactions of urea indicate definitely that urea in these cases, at any rate, should be assigned the structure $NH : C(OH).NH_2$. In the second place, E. A. Werner, in a series of publications extending over a number of years [20] has brought for-

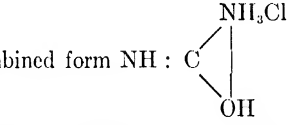
[20] Summarized in "The Chemistry of Urea," by E. A. Werner, Longmans, Green and Co., London, 1923.

ward considerable experimental evidence to show that urea might best be represented by the following structures:  .

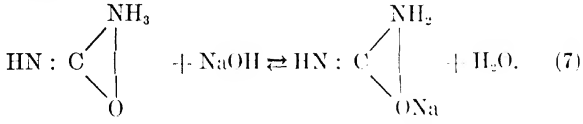
It is impossible to enter into the detailed evidence which he presented in support of this view, but the conclusions obtained for the reactions involving the hydrolysis of urea may be given. In the presence of acids (hydrochloric acid for example), the reaction is assumed to take place according to the following equations:

 $+ HCl = NH_4Cl + (HNCO \rightleftarrows HOCN)$ (5)

$$(HNCO \rightleftarrows HOCN) + H_2O + HCl = NH_4Cl + CO_2 \quad (6)$$

Cyanic acid (formulated as an equilibrium mixture of the two tautomeric forms) is very rapidly hydrolyzed. The velocity of the complete reaction is therefore controlled by the rate of dissociation of the urea, since both the products of the dissociation, ammonia and cyanic acid, are removed as rapidly as they are formed. In the presence of hydrochloric acid, about half of the urea exists in the combined form  . Since only free urea reacts according to equation (5), the velocity of this reaction should decrease with increase in concentration of hydrochloric acid. This decrease is appreciable only if the concentration of the hydrochloric acid is greater than normal. With a weak acid, more free urea would be present in solution, but, on the other hand, the ammonia would not be removed (fixed) so rapidly from the sphere of action. These opposing tendencies then result in the facts observed experimentally that initially and up to a certain point, the rate of decomposition of urea is greater in the presence of weak acids than of strong, but that in the former case the time for complete decomposition of the urea is greater than in the latter.

With alkali, urea reacts as follows:



This reaction takes place to a small extent only. At sodium hydroxide concentrations less than normal, practically all the urea is present in the free state. Because of the dissociation of the urea and the sodium cyanate formed, the velocity of the whole reaction depends on the rate of hydrolysis and the rate of decomposition of cyanate (ammonium or metal salt).

In general terms, these reactions of urea depend primarily upon its dissociation. Below a certain temperature, this dissociation is negligible, even in the presence of acid or alkali. Above this temperature it may be mentioned that the presence of cyanate has frequently been observed in solutions of urea.

The main fact which develops from these considerations is that, whatever structure may be assigned to urea, the hydrolysis unquestionably will prove to be of greater complexity than has heretofore been assumed in the simple formulation.

The chemical reaction in which sucrose is hydrolyzed to form glucose and fructose is extremely interesting, not only from the point of view considered earlier in this chapter but also as a reaction which is influenced by definite enzyme preparations. This enzyme action has been studied extensively and will be considered more in detail in later chapters. There are, however, a number of chemical changes which occur in living matter which may well be considered briefly in this connection. Some of these changes are known to be influenced, if not controlled, by enzymes in the living organisms, others are presumed to be so influenced or controlled. The chemical changes referred to include the various transformations undergone by carbohydrates, both in plants and animals. These changes comprise one of the most important of the group of metabolic and catabolic processes which occur in nature. Special interest is lent to certain of these changes at the present time, because of the importance of the discovery of insulin and probably analogous preparations in the treatment of disturbed carbohydrate metabolism in human beings, and the occurrence of these substances in so many

vegetable and animal materials. Although the method of action of *insulin is not known at present, the chemical changes undergone by carbohydrates under certain conditions which may be related to the* conditions of action of the insulin, may be of interest, especially since there is a strong probability that enzyme or similar actions are involved in some stage of the insulin action.

Although the formula of glucose apparently does not indicate a high degree of complexity, its behavior with various reagents shows it to be an extremely reactive substance capable of giving a large number of products when subjected to different treatments. This may be shown by a quotation from a paper by Nef on "Dissociation Reactions in the Sugar Group": [21] "After nine years of extraordinarily difficult work, it has been possible finally to explain completely and in an extremely simple way the behavior of all possible simple sugars in solution in caustic alkali toward oxidizing agents such as air, hydrogen peroxide, mercuric-, cupric-, and silver oxides. For this it was necessary above all to understand the behavior of this group of substances toward caustic alkali alone; in the last six years I studied this question continuously. The second paper on "Dissociation Reactions in the Sugar Group," published three years ago, treated of this subject exclusively; the most important result obtained was the proof that in a weak solution of caustic alkali any ordinary variety of sugar forms finally an equilibrium, in which theoretically not less than 116, practically however as was found, only 93 different substances take part. This equilibrium mixture, in the absence of oxidizing agents, is changed by conversion of the different sugars irreversibly into C_3-, C_4-, C_5-, C_6- saccharic acids; if on the other hand, air or any other oxidizing agent is present, by oxidation of the 47 sugars present, a terrifying mixture is formed of carbonic acid, formic acid, glycollic acid, oxalic acid, *dl*-glyceric acid, isomeric trihydroxybutyric acids (4), tetrahydroxyvalerianic acids (8), and pentahydroxycaproic acids (8), with whose sepation and quantitative isolation I have been busy for 9 years."

In view of the evident possible complexity of the changes which the decomposition of glucose may involve, it seems almost hopeless to give a satisfactory account of the subject in the limited space available. An attempt, however, will be made to illustrate a few points. In the first place, the ultimate products in the complete

[21] J. U. Nef, *Lieb. Ann.*, *403*, 204 (1914).

decomposition are carbon dioxide and water. In the second place, although a great number of products short of these ultimate products are possible, in any living organism which metabolizes glucose, only a very limited number of such intermediate products have been found in any amount. Further, the nature of the products are different frequently in the different organisms. Although experimental methods are not quite satisfactory and the study of the subject may be said to have been only begun, the results indicate definitely that the course of the changes, hydrolyses or oxidations or both, in the degradation of glucose may be quite different in the various cases. No one case has yet been worked out completely. A number of different workers have developed different schemes showing the probable changes occurring. In place of attempting a critical summary of the present status of the subject, the views of a few individuals will be presented. Nothing will be said of the possible actions of enzymes here; only the chemical transformations observed or assumed to occur with glucose will be considered.

The changes which are undergone by glucose in the animal body have been summarized by Dakin.[22] It is pointed out that the disappearance of glucose is not always due to its breakdown, but that polysaccharides may be formed. Active glucose metabolism only seems to occur in the presence of living or surviving cells. Glucose with a number of tissues is transformed quantitatively into lactic acid. Considerable evidence is at hand that in this reaction glyceric aldehyde is formed first, probably also dihydroxyacetone, that these lose a molecule of water to form pyruvic aldehyde which then takes up a molecule of water to form the lactic acid. The evidence for and against this view is presented by Dakin. Apparently it is the most satisfactory picture of these changes as far as they go. The fact that a very small amount of ethyl alcohol has definitely been found in muscle is of interest.[23]

The decomposition of glucose by yeast has been studied extensively. Perhaps the best or at least the most satisfactory picture of the changes is that developed by Neuberg.[24]

[22] H. D. Dakin, "Oxidations and Reductions in the Animal Body," Longmans, Green and Co., London, 1922, Chapter IV; "The Carbohydrates."

[23] A. E. Taylor, *J. Biol. Chem.*, *15*, 217 (1913).

[24] A review of the work of C. Neuberg in this field was published by W. Fuchs, *"Sammlung chemischer und chemisch-technischer Vorträge,"* 27, 1-48 (1922).

glucose ($C_6H_{12}O_6$)
↓ $-2\ H_2O$
methylglyoxalaldol ($CH_3.CO.CHOH.CH_2.CO.CHO$)
↓
methyl glyoxal (pyruvic aldehyde) (CH_3COCHO)
↓ Cannizzaro reaction

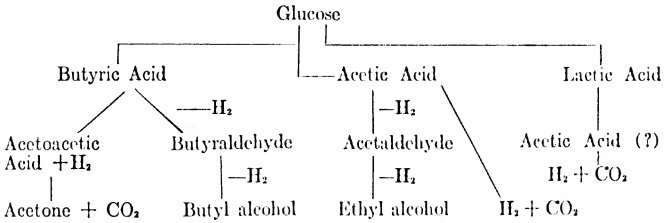
glycerin + pyruvic acid
↓
acetaldehyde + carbon dioxide
acetaldehyde + methylglyoxal
↓ $+ H_2O$
pyruvic acid + ethyl alcohol

The first two steps given above are hypothetical; the third is extremely probable, some form of the Cannizzaro reaction taking place. The changes undergone by pyruvic acid are fairly certain. As indicated in the first series, glycerin, acetaldehyde, and carbon dioxide are the products. The second series shows that as soon as some acetaldehyde is formed it reacts with methyl glyoxal to form ethyl alcohol and pyruvic acid. The pyruvic acid then decomposes again to form acetaldehyde and carbon dioxide, and the acetaldehyde goes through the various stages of the reaction again. The acetaldehyde would then be acting as catalyst in the sense described in Chapter II. Under these conditions, the products obtained would be ethyl alcohol and carbon dioxide with only a small amount of glycerin produced at the very beginning of the reaction. If, however, a reagent such as sodium bisulfite were added which removed the acetaldehyde from the sphere of action, it could not act as catalyst as shown. Glycerin, acetaldehydebisulfite compound, and carbon dioxide would then be obtained as the products of the fermentation. With alkali, the acetaldehyde may undergo a Cannizzaro rearrangement to form acetic acid and ethyl alcohol.

The formulations just given while not final in any sense form an interesting picture of some of the changes which may occur and explain a number of the facts which have been observed.

In place of going into the views of a number of additional workers, only the suggestions put forward recently by Speakman will be given. As a result of his experimental studies, mainly on the butyl alcohol fermentation process of glucose, Speakman [25] suggested the following provisional scheme of fermentation:

[25] H. B. Speakman, *J. Biol. Chem.*, *58*, 395 (1923).



This formulation introduces the view that the organic acids are derived from glucose by cleavage and oxidation. In general the initial products from hexoses are taken to be $C_4 + C_2$ and $C_3 + C_3$ compounds.

The results which are given on the breaking down of glucose under the different conditions are not satisfactory in so far as they do not permit of a complete understanding of the course of the chemical changes which occur. Almost every worker who has studied the problem has modified some existing scheme. This is of course due in part to the complexity of the reactions and in part probably to the different influences which added substances, aside from living organisms, may exert. The oxidation of glucose by hydrogen peroxide in the presence of phosphate may be referred to in this connection.[26] Glucose in aqueous solution was not changed by hydrogen peroxide or disodium hydrogen phosphate alone, but was oxidized completely to carbon dioxide and water if both were added. The hydrogen ion concentration had little influence on the action over rather a wide range. The amount of the phosphate seemed to be the dominating influence. The use of sodium carbonate and bicarbonate in no way produced a similar action on the glucose. No evidence was obtained of the formation, either intermediate or otherwise, of a hexosephosphate as may perhaps have been expected.

These last results are extremely interesting and suggestive, although it is still too early to attempt to evaluate their significance in the general scheme of glucose degradation. This degradation involves both hydrolysis and oxidation reactions. The consideration of glucose oxidation from the valence electron point of view

[26] W. Löb and G. Pulvermacher, *Biochem. Z.*, *29*, 316 (1910); W. Löb and S. Gutmann, *Biochem. Z.*, *46*, 288 (1912); W. Beysel and W. Löb, *Biochem. Z.*, *68*, 368 (1915); and especially E. J. Witzemann, *J. Biol. Chem.*, *45*, 1 (1920).

and the electric potentials produced may be mentioned in this connection.[27]

This leads to a brief consideration of oxidation (and reduction) reactions in so far as they may be included in their relation to enzyme reactions.

Three general types of oxidizing enzymes may be described briefly: (1) Oxidases, which increase the velocity of the oxidation of a number of different organic substances, such as alcohols, aldehydes, phenols, amines, etc. (2) Peroxidases, which increase the velocity of the oxidation of similar substances in the presence of hydrogen peroxide. (3) Catalases, which increase the velocity of the decomposition of hydrogen peroxide, gaseous oxygen being evolved.

The experimental work with the various oxidizing enzyme preparations has not as yet reached the stage where generalizations can be developed as a result of the study of enzymes from different sources and the various reactions which they catalyze, or specific substances whose oxidation reactions they catalyze. There is considerable variety in the different substances which may be oxidized. The time seems to be ripe for a careful and systematic study of these oxidation reactions and the enzymes which are connected with them.

The reactions whose velocities are increased by oxidases depend upon the oxidation of a substrate without the addition of any other substance. The oxidation must involve, therefore, oxygen of the air or the reduction of some atom of the oxidase preparation or substrate mixture. In general, the former seems in every way to be the more likely assumption. This would mean then that the reactions are auto-oxidation reactions catalyzed by the enzyme preparation. A review of "Autoxidation of Organic Compounds" was published some years ago.[28] For the historical development of the subject and enumeration of a great number of reactions reference may be made to the publications of C. Engler and Weissberg [29] and of J. W. Mellor.[30] The most probable course of these reactions indicates that molecular oxygen is added to the substance being

[27] J. Stieglitz, *Proc. Inst. Medicine of Chicago, 1,* 41 (1916-7).
[28] K. G. Falk, *School of Mines Quarterly, 29,* 15 (1907).
[29] "Kritische Studien uber der Vorgänge der Autoxydation," 1904.
[30] "Chemical Statics and Dynamics," pp. 304-339.

oxidized, and that this "peroxide" may then decompose, part of the oxygen being given off readily and being able to bring about further oxidations. It is difficult to state the exact part taken by the oxidase in the reactions.

In this connection the isolation and study by F. G. Hopkins [31] of a cell constituent capable of acting as an oxidation-reduction system may be mentioned. This constituent, called glutathione, is a dipeptide of d-glutamic acid and l-cysteine and is heat stable. The oxidized form contains the disulfide cystine group, the reduced form the hydrosulfide cysteine group. The reaction involved is the SH and S-S reciprocal change. The substance is oxidized and reduced with any suitable agent. It is not specific in any sense. It is obtained in yields of from 0.01-0.02% from fresh tissue or yeast. "The facts suggest that coexisting in living tissues with the specialized enzymic mechanism for oxidations and reductions, materials in some close association with structural elements are oxidized, anaërobically or aërobically, with the coagency of the sulfur group in glutathione."

The peroxidase reactions are similar to the oxidase reactions except that in place of the oxygen of the air taking part in the reaction, hydrogen peroxide is required. It would appear superficially that the peroxidase enzymes are not as active, if this very loose and unscientific term may be used, as the oxidase enzymes which increase the velocities of the reactions in which the molecular oxygen of the air is involved, while the former need hydrogen peroxide, which as a rule oxidizes substances more rapidly. The part played by the peroxidase is also not definite. That the reaction takes place in steps involving the formation of intermediate addition compounds appears to be unquestioned, but further than this, it is difficult to go at present.

A beginning has been made in the last years in placing the oxidation-reduction relations on a firm theoretical basis and developing experimental methods and reagents to make possible comparative studies of various systems. The publications of W. M. Clark may be referred to especially in this connection.[32] The general method

[31] F. G. Hopkins, *Biochem. J.*, *15*, 286 (1921); F. G. Hopkins and M. Dixon, *J. Biol. Chem.*, *54*, 527 (1922); J. H. Quastel, C. B. Stewart and H. E. Tunnicliffe, *Biochem. J.*, *17*, 586 (1923).

[32] W. M. Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Company, Baltimore, 1922, especially Chapter 16 and references; W. M. Clark,

is to determine the difference in potential between a normal hydrogen electrode, selected as a convenient standard of reference, and a solution containing a substance and its oxidation product both in determinable concentrations, at controlled hydrogen ion concentrations. The substance and its oxidation product must be in equilibrium with each other and both oxidant and reductant should be present in finite ratio. Pairs of such substances which have been investigated include quinone-hydroquinone, 1-naphthol-2-sulfonic acid indophenol and its reduction product, indigo-indigo white, methylene blue-methylene white, etc. The relation between the reducing potentials and the hydrogen ion concentrations of the solutions were studied both theoretically and experimentally.

In view of the fact that these studies have only been carried out with comparatively simple organic substances and not with the more complex materials encountered in enzyme investigations which in addition are as a rule far removed from equilibrium conditions, this brief reference to the most recent advances in the exact study of oxidation-reduction relations may suffice. At the same time the study of such a system as glutathione and its reduction product may well be possible at the present time and yield results of interest.

With catalase; the present status of the study is not very satisfactory. The decomposition of hydrogen peroxide appears to be similar to the action of a number of colloidal metals in increasing the velocity of the same reaction, but aside from the probability of the formation of intermediate compounds of some type, nothing can be said with reference to the reactions.

B. Cohen and M. X. Sullivan, "Studies on Oxidation-Reduction," *U. S. Public Health Service, Reprints Nos. 823, 826, 834, 848* from *Public Health Reports, 1923*, pp. 443-455, 668-682, 933-958, 1669-1718. Cf. also F. S. Granger and J. M. Nelson, *Jour. Amer. Chem. Soc., 43*, 1401 (1921); V. K. LaMer and L. E. Baker, *Jour. Amer. Chem. Soc., 44*, 1954 (1922); and others.

IV.—Physical Properties Common to Enzyme Preparations

In the description of various preparations which are classed as enzyme preparations, it has been found that a number of properties are in a sense common to all. That is to say, these different preparations from various sources have certain reactions or properties which, to a certain extent are similar. These similarities were originally observed in a purely empirical manner. Gradually, as a result of observing them, it became customary to define or describe an enzyme preparation as possessing certain of the properties which had been found to be more or less common to those previously studied.

In this and the following chapters some of these properties will be discussed. No attempt will be made to give the experimental details with every enzyme preparation which has been studied, or even to give a complete list of such studies. Only the general relations will be presented with as much of the individual experimental evidence as appears necessary to make clear the nature of the results.

It will be noted that in the headings of the chapters a distinction has been made between the physical and the chemical properties common to enzyme preparations. It is impossible to draw a definite line of demarcation between the two sets of properties. Any distinction or classification of this sort which is made is to a great extent arbitrary and dependent upon the personal point of view of the one making this distinction. This will appear more clearly in the following pages.

In working with enzyme preparations the first characteristic property met with is the colloidal property. Practically all enzymes exist as colloids. They do not dialyze, or dialyze extremely slowly through collodion or other suitable membranes. This property or characteristic may, perhaps, be defined more closely. J. M. Nelson

and D. P. Morgan[1] showed that with collodion membranes and yeast sucrase, the thickness and porosity of the membrane could be modified in a quantitative manner by varying the alcohol content of the collodion solution, the thickness of the liquid film, the time of drying, etc. In this way collodion films of reasonable strength were obtained at will which either permitted the enzyme to pass freely but retained certain impurities, or which did not permit the enzyme to pass at all. An important fact was pointed out by Willstätter, Graser and Kuhn,[2] namely, that collodion membranes or bags in dialysis are at times not as indifferent and harmless as is generally accepted, because of the readily hydrolyzable and reducible cellulose nitrates present. On the other hand, fish bladders which were also used were found not reliable because of the possibility of protein material from them going into the enzyme solution. Another factor which must be considered in carrying out dialyses is the composition of the water against which the solution or mixture in question is dialyzed. This was shown strikingly in some experiments with banana extracts.[3] Dialyzing these extracts in collodion bags against tap water resulted in the formation of gels. This was found to be due to the calcium content of the tap water which also showed a pH of 7.0. Dialyzing the extracts against distilled water of pH 5.0, or distilled water brought to a pH of 7.0, no gel was formed, but if the calcium salt was added, the gel was produced. Dialyzing the gel against distilled water resulted in its disappearance. Direct addition of very small amounts of calcium salts to the banana extracts at pH 5.0 produced no gel, at pH 7.0 a gel was produced within a few minutes. Evidently long continued dialysis against water of suitable alkalinity containing very small amounts of calcium salts resulted in the formation of gels similar to those formed with larger (though still very small absolute) amounts of calcium salts by direct precipitation.

These results indicate in the first place that possibilities of error exist in the use of various membranes for dialysis; in the second place that membranes may be prepared and standardized which will permit of more accurate differentiation and separation of the substance present in enzyme materials from different sources; and in

[1] J. M. Nelson and D. P. Morgan, *J. Biol. Chem., 58,* 305 (1923).
[2] R. Willstätter, J. Graser and R. Kuhn, *Z. physiol. Chem., 123,* 1 (1922).
[3] G. McGuire and K. G. Falk, *J. Gen. Physiol., 4,* 437 (1921-1922).

the third place, that the water or solution which is used as the external liquid may influence profoundly the enzyme and other properties of the solution under investigation because of its composition.

In the study of enzymes, advantage is taken of the non-dialyzing property in separating the active enzymes as far as possible from accompanying inactive material which does dialyze, and also in preparing certain enzyme extracts by means of salt solutions, for the purpose of subsequently removing the salt. It may be stated that although there is scarcely an enzyme preparation which has not been treated in this way, it is unfortunately true that the direct information with regard to the chemical nature of enzymes or of the conditions for their actions, which has been gained up to the present as the results of dialysis studies, is practically negligible.

The questions involved in the dialysis or passage of enzymes through membranes apply as well to cells and cell structures. Enzymes at times have been grouped as endocellular and intercellular, as those present within cells and those found in the liquid outside the cells. Aside from the conditions of action of the enzymes in the living organism, the occurrence of enzymes as indicated is an important consideration in their study in the laboratory. That is to say, it is necessary with the endocellular enzymes, to obtain the enzyme material in solution or otherwise free from the cells without destroying the enzyme actions. For example, Willstätter and Racke [4] showed that the carbohydrate hydrolyzing enzymes can be separated from yeast and brought into solution by two methods; either by the action of added enzymes (first by pepsin or trypsin to decompose protein material and then by tannase or malt diastase to decompose the insoluble carbohydrates and so make possible the solution of the enzyme); or by mechanical means, not only tearing and grinding the cells but completely destroying the cell structure. This last is accomplished with greater difficulty than has been assumed heretofore by a number of investigators.

The distribution of trypsin in gelatin suspension between the outside liquid and the protein at different hydrogen ion concentrations has been shown by Northrop [5] to follow the membrane equilibrium equations developed by Donnan. In solutions more acid than pH 10.2, trypsin behaved as a univalent positive ion, in more alkaline

[4] R. Willstätter and F. Racke, *Lieb. Ann.*, *427*, 111 (1922).
[5] J. H. Northrop, *J. Gen. Physiol.*, *6*, 337 (1923-1924).

solution as a univalent negative ion. The trypsin ions apparently diffused readily into and out of the gelatin particles but their equilibrium concentrations under definite conditions of acidity, etc., were different in the gelatin and in the outside solution. These results are quoted here as indicating that under suitable conditions enzyme material may penetrate protein particles. They will be taken up again in connection with the consideration of the chemical properties of enzymes and the mechanism of enzyme action.

Many substances of biological or biochemical origin do not dialyze through membranes ordinarily and it might be considered that this colloidal property of enzyme preparations is due to the fact that they are obtained from such biological materials and retain or possess their properties, while the actual enzymic action is in itself unconnected with the colloidal nature of the substance. This brings up at once a question which has been considered frequently, especially by W. M. Bayliss in his monograph on "The Nature of Enzyme Action,"[6] whether the predominating feature of enzyme action is to be taken to be the property of adsorption possessed by enzyme preparations largely because of their colloidal nature, or whether the reactions may be considered to be more chemical in character. Bayliss inclines to the view that adsorption is the prime factor involved. His views may be summarized briefly here, although reference must be made to his monograph for the detailed evidence.

Bayliss gave reasons for believing that the action of enzymes in general must be regarded as exerted by their surfaces, resulting in the formation of a colloidal adsorption compound with the substrate. He states that "By surface condensation the reacting constituents are brought into intimate contact and accelerated by mass-action. Whether chemical combination between enzyme and substrate occurs in any stage of the process is not yet decided. Direct experimental proof exists that enzymes act by their surfaces in liquids in which they are completely insoluble." In fact, Bayliss[7] appears to have been the first to advocate the view that the rate of change is a function of the degree of adsorption in the different stages of the reaction. The increased rate of reaction is considered to be due to the increase in active mass (concentration)

[6] IV Edition, published by Longmans, Green and Co., 1919.
[7] W. M. Bayliss, *Biochem. J.*, *1*, 175 (1906).

owing to concentration on the surface. In order to account for the fact that certain substances are adsorbed by a certain surface, the chemical nature of the surface in question must be considered. This is especially true when considering specificities of enzyme action, markedly so with optical isomers, where in a partial analogy to the "lock-and-key" simile of Emil Fischer (which will be taken up again later) "it may be said that the chemical *configuration* of the surfaces of contact, or the molecular shape of the constituents of the surfaces, are potent factors in determining the possibility of intimate contact between them." He also points out that adsorption may be followed by chemical combination with the surface of the enzyme, although there is no evidence that it occurs. Following the adsorption of the substrate by the enzyme, an equilibrium is attained with the products of reaction formed from the substrate.

The adsorption point of view as advocated by Bayliss and superficially outlined here lays emphasis on the physical phenomenon of adsorption as the controlling factor in changing the velocity of the chemical reaction. At the same time, Bayliss considers the possibility of the chemical reaction with the surface being the predominating factor, but prefers the former view, pointing out, however, that at the same time, the physical properties of a surface are conditioned by its chemical nature.

The writer prefers to look upon enzyme action as essentially chemical in character. The reactions would follow the general laws of chemical reactions, and if the mechanism outlined in Chapter II be accepted, the theory of intermediate compound formation should be applicable to the reactions in question also. The isolation and identification of intermediate or primary addition compounds between substrate and enzyme has not been successful as yet, although the view that they are formed is widely accepted, but at the same time it must be remembered that the nature of the enzyme molecules is quite obscure. With regard to adsorption phenomena, it might be considered that the purely physical phenomenon takes place first and is followed by chemical reaction with the enzyme as catalyst. The enzyme preparation might also adsorb or be adsorbed by a substance which is not changed by it or whose velocity of transformation is not affected by it. At the same time, such views should include the possible, and even probable, relation of the chemical properties of the reacting constituents upon the forma-

tion of adsorption compounds. The point of view adopted by Langmuir and by Harkins in connection with the orientation of liquids on surfaces carried over to adsorption, which was given in the first chapter, seems to be especially relevant. Bancroft[8] emphasized the same fact in an address on "Contact Catalysis" where he considered the increase in concentration in its effect on catalytic action, when reacting substances are adsorbed at the surface of a catalytic agent as relatively unimportant in most of the cases studied hitherto. He did not speak of enzymes as such, but only of catalytic actions in general. Reference may also be made in this connection to a General Discussion[9] held by the Faraday Society on "Heterogeneous Reactions" in which the newer views on "Chemical Reactions on Surfaces" based mainly upon orientation of molecules on surfaces and their spatial arrangements on these surfaces were presented.

E. F. Armstrong and T. P. Hilditch[10] arrived at conclusions analogous to those given, in a paper on "Catalysis on Solid Surfaces." Comparing the hydrogenation of organic substances (such as unsaturated fatty acids) to enzyme actions, they stated: "In each case, the catalyst (enzyme or reduced nickel) unites primarily with the organic compound about to undergo change (hydrolyte or unsaturated glyceride), the complex so formed being decomposed by the other component of the interaction (water or hydrogen). In each case, moreover, action takes place entirely at the surface of minute particles and the activity of the catalyst depends entirely on the production of maximum surface and the avoidance of impurities likely to destroy or dirty this surface."

The following evidence points in the same direction:[11] Animal charcoal adsorbed scarcely a trace of glycine, but it adsorbed a little alanine and a comparatively large quantity of leucine. Similar variations were observed with polypeptides, even isomeric substances being adsorbed to very different extents by charcoal. The amounts adsorbed at various dilutions varied according to the adsorption law just as with enzymes. The adsorptive power of charcoal differed from that of enzymes in not being influenced by

[8] Presidential address, 1920, American Electrochemical Society.
[9] *Transactions of the Faraday Society, 17,* 607-675 (1922).
[10] *Proc. Roy. Soc. London (A) 96,* 137, 322 (1919).
[11] E. Abderhalden and A. Fodor, *Fermentforschung 2,* 74, 225 (1917-1918); *Kolloid-Z. 27,* 49 (1920).

changes in the hydrogen or hydroxyl ion concentration of the solution.[12] Since it was found that adsorption can occur with inactivated enzyme solutions, it was concluded that while adsorption of the substrate by the enzyme precedes fission, the latter process does not necessarily follow the former. Evidence for this is also shown by the behavior of glycyl-l-leucine, which, at 0°, is adsorbed by yeast extract, but not decomposed.

It seems simpler from the chemical point of view to lay the stress for enzyme actions, in the cases in which adsorption obviously takes place as well as in those in which it is not so apparent, on the chemical actions between substrate and enzyme. With regard to the reaction velocity equation frequently taking the form of the adsorption equation, in view of the complexity of the reactions, especially with insoluble colloids, the application of kinetic equations in their simple form upon any basis appears to possess somewhat questionable validity. This question will be considered in greater detail in Chapter VII.

The problem of adsorption and the formation and precipitation or coagulation of colloid material upon the addition of suitable reagents has always been of considerable interest especially in connection with substances obtained or derived from living matter. Such relations have frequently been described for enzyme materials as already indicated. At the same time, the results obtained with enzymes have been, in the main, sporadic, different investigators studying the behavior of single enzyme preparations or of isolated reagents with a number of enzyme preparations. During the past five years, Willstätter and his co-workers have published the results of systematic studies on the adsorption and precipitation of a number of enzymes with various reagents (or perhaps more suitably, coagulents). The object in view was to obtain the enzyme substance free from inactive material and in as great a state of purity as possible. The ordinary methods of preparative organic chemistry, such as distillation, solution and recrystallization, etc., are not applicable because of the nature of the materials. Further, methods such as may be employed for more complex materials (sugars, peptides, etc., not to mention starches or proteins) which

[12] In other cases, however, the adsorptive power of carbon was found to be influenced not only by the hydrogen ion concentration of the mixture, but also by the previous treatment to which it may have been subjected. Cf. for example, N. K. Chaney, A. B. Ray, and A. St. John, *Ind. Eng. Chem. 15*, 1244 (1923).

may involve salt or definite compound formation with acids or bases, precipitation with salts of heavy metals, etc., can only be used with great caution because of the inactivation of the enzymes in many cases upon comparatively mild treatments. It follows, therefore, that there remain only methods such as might be included in the adsorption processes, where partial precipitations and separations may be affected by the addition of reagents which are comparatively innocuous to enzymes. The practical difficulties involved in the control of the experimental conditions in such studies appear to have delayed the systematic application of these methods. The success which Willstätter attained in this field, therefore, marks a definite step forward in the experimental study of enzymes.

The problem, in general terms, consists of two parts, the action of the adsorbant on the enzyme material to form an insoluble precipitate, and the elution of the enzyme with a suitable solvent from the precipitate. The adsorption process should be as selective as possible. As a rule, the adsorbant takes out of the (colloidal) solution, in addition to the enzyme, varying amounts of foreign material.

Michaelis and Ehrenreich [13] had shown a number of years before that kaolin adsorbs basic bodies from solutions, and aluminium hydroxide acid bodies. These studies had not extended so far as to show specific adsorptions with enzymes. Willstätter and his co-workers worked out the conditions for the specific actions of the adsorbants in purifying the enzymes. Each enzyme material has to be considered separately, and in some cases, after partial purification the adsorption properties of the enzyme were modified profoundly. The enzymes studied included yeast sucrase, plant peroxidase from different sources, saliva diastase, pancreas amylase, trypsin, and lipase, and some seed lipases.

The two main adsorbing materials used were kaolin and aluminium hydroxide, the former taking out the basic materials, the latter the acid. By carrying out the adsorptions in solutions of different acidities or alkalinities, or in solutions containing alcohol or acetone, the reactions could be controlled still farther. The smaller the amount of adsorbant used, the greater was the selective action and therefore the state of purity of the enzyme material. Purified sucrase preparations were obtained from autolyzed yeast by several

[13] L. Michaelis and M. Ehrenreich, *Z. physiol. Chem.*, *56*, 18 (1908).

methods. Successive adsorptions by kaolin and by aluminium hydroxide gave satisfactory products. Sucrase was extracted from the precipitates best by diammonium hydrogen phosphate in 0.05-1% ammonia solution (2.4-2.5 mols NH_3 to 1 mol H_3PO_4). Arsenic or citric acid could be used in place of the phosphoric acid. These phosphate solutions have found general applicability in such elutions. If desired, the phosphates can be removed from the extracts either by dialysis or by precipitation with magnesia mixture. It was found that under certain conditions, sucrase could be eluted from the aluminium hydroxide precipates by sucrose solutions. This is significant because of the view held widely that a substrate protected its enzyme from decomposition to a certain extent, presumably because of being combined with it. This simple view, however, is insufficient to account for the facts observed. For example, sucrase was eluted slowly and incompletely by sucrose solution from the aluminium hydroxide precipitate; quantitatively and in a few minutes by a 16% sucrose solution containing 1% monosodium dihydrogen phosphate. A phosphate mixture, pH 7.0, acted similarly, indicating that optimum pH for action was not involved. That the reaction was not specific for phosphate was shown by the fact that a citrate buffer mixture, pH 4.5, acted similarly, but an acetate buffer mixture, pH 4.5, even inhibited the solvent action shown by the sucrose solution alone. Also dilute ammonia or sodium carbonate solutions could be used for elution. The most active sucrase was obtained by successive adsorptions on aluminium hydroxide and lead acetate. The presence of phosphate aided in the precipitation with lead acetate, while protein inhibited. Autolyzed yeast after treatment with lead acetate to remove proteins (no sucrase being precipitated), on long standing and renewed treatment with lead acetate gave a precipitate containing sucrase. Possibly the nucleoprotein present was decomposed on standing, and the nucleic acid precipitated by the second lead acetate treatment. This illustrates the part played, and aid given at times, by foreign bodies in adsorption. Also, the sucrase was adsorbed readily from partially purified solutions by kaolin from which it was eluted best by dilute sodium carbonate solution, but from crude yeast autolyzates only under certain conditions. The behavior when the electrochemical nature of the adsorbant was changed was shown with sucrase by the fact that treatment with aluminium

hydroxide gave a concentration of enzyme 100 times as great as the original material but still containing 65-75% yeast gum. Treatment with kaolin left all of the yeast gum in solution. The enzyme could then be eluted with very dilute alkali. The most rapid method of obtaining the sucrase in a high state of purity was found to consist in removing proteins with N/20 acetic acid, then diluting 20 to 40 fold and adsorbing with kaolin. Elution with very dilute ammonia yielded the desired product.

These procedures serve to illustrate the various methods which may be used in the purifications. The separation of different enzymes from each other was worked out in the case of pancreas for amylase, trypsin, and lipase. The lipase here shows the most marked acid properties, the trypsin the strongest basic properties. The former is readily adsorbable by aluminium hydroxide and by kaolin, the latter by kaolin, while the amylase is indifferent in aqueous solution when partially purified toward both electropositive and electronegative adsorbants. The method of separating these three enzymes from each other appears, therefore, to be quite simple in principle; first aluminium hydroxide adsorption to remove the lipase, then kaolin adsorption to remove the trypsin, leaving the amylase in solution. The practical working out of this procedure is not so simple, however, because of the presence of foreign substances which modify the adsorption properties of the three enzymes to some extent. A description of the separation of the three enzymes from each other may serve to show the general scheme which can be employed. Treatment of the aqueous or aqueous-glycerin extract of the pancreas gland with aluminium hydroxide removes most of the lipase, about one-third of the amylase, and some of the trypsin. The accompanying foreign bodies take the latter two enzymes into the precipitate, but, on the other hand, retain them to a greater extent in the elution process. Eluting the precipitate with alkaline phosphate in aqueous-glycerin solution (2 1/3 NH_3 to 1 H_3PO_4) results in extracting two-thirds of the lipase, 3% of the total trypsin present initially in the gland, and only slightly more of the amylase. Repetition of the aluminium hydroxide precipitation and elution gives a quantitative separation of the lipase, in a concentration about thirty times as great as in the original pancreas. The trypsin-amylase mixture can be separated by two successive treatments with kaolin, the trypsin being precipitated, although with

considerable loss. The amylase, after partial purification in this way, is indifferent toward both electropositive and electronegative adsorbants in aqueous solution. In 50% alcoholic solution, however, it is adsorbed by aluminium hydroxide, the alcohol evidently making the acid properties of the associated amphoteric substances more pronounced.[14] After elution with water, it is possible only to adsorb a small part of the amylase under the same conditions as before, the foreign substances showing the adsorbable properties evidently having been removed in the treatment. It is interesting to note that the pancreatic amylase after purification showed the weakest adsorptive power of any of the enzymes studied. In direct contrast is the behavior of sucrase which is more readily adsorbed the greater the state of purity of the enzyme.

It may be mentioned that the pancreatic lipase can be purified still more by adsorption on indifferent organic substances, such as cholesterin and tristearin, which act more selectively here than do the aluminium hydroxide and kaolin.

The studies on peroxidase have also given important results.[15] Peroxidase occurs in the plant cell partly in solution in the cell juice in which form it can readily diffuse out, and partly in the protoplasmic bodies from which it can only be liberated after the complete destruction of the cell structure. All of the enzyme can be obtained, for example, from turnips or horseradish, by treatment with sodium bicarbonate. The enzyme can be obtained in concentrated form by various adsorption procedures. The best product was obtained as the result of four adsorptions by aluminium hydroxide, three by kaolin and one by tannin, in the following order, kaolin, aluminium hydroxide, tannin, kaolin, aluminium hydroxide, etc. The basic properties predominate in peroxidase, the addition of alcohol being necessary therefore in the aluminium hydroxide adsorption. The kaolin adsorptions may be carried out either in aqueous-alcoholic or in acetic acid solution, those with tannin in alcoholic solution containing acetic acid. The enzyme is brought into solution again from the aluminium hydroxide precipitates by water containing carbon dioxide, from the kaolin

[14] This fact was observed with aminoacids and peptides, R. Willstätter and E. Waldschmidt-Leitz, *Ber., 54 B,* 2988 (1921). Cf. also K. G. Falk and J. M. Nelson, *Jour. Amer. Chem. Soc., 34,* 830 (1912).

[15] Cf. especially the third paper on this topic; R. Willstätter and A. Pollinger, *Lieb. Ann., 430,* 269 (1923).

precipitates by dilute ammonia. The tannin-peroxidase precipitate is decomposed by dilute ammonia but both products remain in solution so that a treatment with kaolin is necessary to remove the enzyme from the tannin.

The properties of the adsorbing reagents are of paramount importance in the separations described. These properties are to a great extent dependent upon the method of preparation of the reagent. For instance, the following forms of aluminium hydroxide were described:

(A). Precipitation from the sulfate with an excess of concentrated ammonium hydroxide at 60° followed by long boiling, and washing by decantation until the ammonia was completely removed.

(B). Similar method to (A) but without long boiling.

(C). Hydrolysis with dilute ammonium hydroxide followed by dialysis.

(D). Sodium aluminate plus carbon dioxide.

Product (B) gives probably the most useful adsorbant; although product (A) containing 4% sulfate is almost as good for preparative work as the neutral product. The different products show different solubilities in sodium hydroxide and hydrochloric acid, different behaviors on drying, different actions in adsorption, etc. Probably a number of definite hydrates are capable of existence. The kaolin may be either boiled with hydrochloric acid and washed until the wash water is neutral toward litmus, or boiled with hydrochloric acid, heated with ammonia and washed, or perhaps best, purified by electro-osmosis.

Although aluminium hydroxide and kaolin show adsorption powers apparently not dependent upon the properties of any one enzyme but rather connected with the chemical properties of more general character, certain adsorbants seem to show specific affinities for definite enzymes. For example, tannin appears to be a specific adsorbant for peroxidase.

The most difficult problem which was encountered in attempting to obtain pure enzyme preparations by adsorption methods was in the removal of substances very closely related to the enzyme material itself and consisting probably of inactivated enzyme, or the progenitors or decomposition products of the enzyme. This problem has not as yet been solved.

The effect of dilution of the enzyme solution on the selective adsorptions is sometimes very striking. Thus, as stated before, the action of aluminium hydroxide on an impure sucrase solution is much more selective in very dilute solution. Not only is the relative amount of the enzyme adsorbed greater in the more dilute solution, but the absolute amount adsorbed is also greater. For the peroxidase solutions, the same relation holds with kaolin but not with aluminium hydroxide, while for lipase it has not up to the present been found to hold at all.

Although it would be possible to enter into much greater details in the description of the various adsorption procedures used and to give the experimental details with the different enzyme preparations and the results obtained, enough has been said to show the principles involved, the methods of overcoming difficulties, and the nature of the results which may be expected. Each enzyme material must be studied separately and the most suitable reagents and conditions for precipitation and elution worked out. At the same time it is clear that these adsorption methods as developed by Willstätter are yielding the most satisfactory enzyme products yet obtained, and offering methods which permit of the separation of the various enzymes from each other.

The following references give most of the work published by Willstätter and his co-workers on this problem since 1918:

General Treatment. *Ber., 55B,* 3601 (1922).

Enzyme Units. *Ber., 56B,* 509 (1923).

Kinetics of Enzyme Actions. *Z. physiol. Chem., 125,* 1 (1923).

Alumina Gels. *Ber., 56B,* 149, 1117 (1923); *57B,* 58 (1924).

Sucrase. *Lieb. Ann.* 425, 1 (1921); *Z. physiol. Chem., 116,* 53 (1921); *123,* 1, 181 (1922); *125,* 28 (1923); *133,* 1, 193 (1924).

Sucrase, Raffinase. *Z. physiol. Chem., 115,* 180 (1921); *127,* 234 (1923).

Maltase. *Z. physiol. Chem., 110,* 232 (1920); *111,* 157 (1920); *115,* 199 (1921); *115,* 211 (1921).

Sucrase, Maltase. *Z. physiol. Chem., 116,* 53 (1921).

Emulsin. *Z. physiol. Chem., 117,* 172 (1921); *121,* 183 (1922); *129,* 33 (1923).

Castor Bean Lipase. *Z. physiol. Chem., 134,* 161 (1924).

Pancreatic Lipase. *Z. physiol. Chem., 125,* 93, 132 (1923); *129,* 1 (1923); *133,* 229 (1924).

Pancreatic and Stomach Lipases. *Z. physiol. Chem., 133,* 247 (1924).

Pancreatic Amylase, *Z. physiol. Chem., 126,* 143 (1923).

Peroxidase. *Lieb. Ann., 416,* 21 (1918); *422,* 47 (1921); *430,* 269 (1923).

Since the colloidal properties are common to enzyme preparations, and since adsorption relations in one way or another can be developed with them as shown in the experimental evidence just presented, it appears advisable to discuss these properties somewhat further and to attempt to show or indicate possible analogies to phenomena observed in other fields of chemistry.

In recent years evidence has been accumulating showing that the chemical reactions which take place with colloids and in general on surfaces may be explained most satisfactorily upon purely chemical grounds. The specificity of the adsorption reactions just described, the fact that kaolin adsorbs basic or electropositive groups and aluminium hydroxide acid or electronegative groups, that substances showing neither basic nor acid properties do not react with, combine with, nor form precipitates with ions or groups of opposite charges, point to chemical reaction and combination in forms similar to those ordinarily considered as underlying these phenomena. The reactions are analogous to reactions studied heretofore in homogeneous media. The laws and regularities involved have not been developed to as great an extent as with the latter, largely because of the experimental difficulties encountered in studying a problem under new or changed conditions. The relations promise in time to be as clear and satisfactory as those for other branches of chemistry and in fact to be based upon the same underlying principles. These views have been developed gradually and by a number of different workers.

In order to show the type of work which underlies the present study of colloids, two investigations will be spoken of. The inorganic relations were outlined in part in a paper by H. T. Beans and H. E. Eastlack on "The Electrical Synthesis of Colloids,"[16] and the studies of J. Loeb summarized in his book on "Proteins and the Theory of Colloidal Behavior"[17] will be given to illustrate a biochemical colloid. Beans and Eastlack studied especially the

[16] *Jour. Amer. Chem. Soc., 37,* 2667 (1915).

[17] Published by the McGraw-Hill Company, New York, 1922.

electrical metal colloid synthesis. They concluded that the formation of the colloid takes place in two steps, first a thermo-mechanical action of dispersion, followed by the formation of a colloidal complex between the highly dispersed metal and certain ions present in the medium. In a pure medium, containing no stabilizing ion, the dispersion behaves as an ordinary suspension and settles out rapidly. The interest in this paper lies in the indication for which experimental proof is given, that a colloid combines in certain ways with other substances or ions present and that the existence of the colloid is dependent upon the presence of these combinations. The experimental difficulties in such an investigation are enormous because of the possible action of minute quantities of foreign substances.

With reference to the experimental work and conclusions of J. Loeb, it may be sufficient to refer briefly to gelatin as a typical example. Loeb found in a series of studies, that gelatin at its isoelectric point ($H^+ = 10^{-4.7}N$ or $pH = 4.7$, is, so to speak, at its transformation point. In more acid solutions it behaves as a salt in which the gelatin complex acts as the cation, in more alkaline solutions it behaves as a salt in which the gelatin complex acts as the anion. A number of different properties such as the electrical conductivity, viscosity, solubility, etc., were followed over a wide range and these relations found to hold. At the isoelectric point the properties of the gelatin passed through a minimum (or maximum) in any of the series of measurements. At that point, the gelatin was present uncombined with acid or salt, or combined with minimum quantities, and showed minimum solubility, etc. It was pointed out clearly that all of the reactions of gelatin could readily be accounted for on this basis, that it reacts chemically either as the positive or negative constituent of a salt depending upon whether the solution is on the acid or alkaline side of the isoelectric point. The chemical reactions of the gelatin complex in the different states (positive, neutral, or negative) might well be different, but the phenomena described appear to be sufficient to account for the observed facts.

Since amphoteric substances play such an important part in biochemical studies, and since they play an important part in enzyme reactions, it may be of interest to enter a little further into some of the theoretical conceptions underlying the simpler amphoteric electrolytes, or ampholytes, since, as stated before, it is believed that

the same fundamental principles or regularities apply to the chemical properties of compounds, whether these are in a crystalloidal or colloidal state. Part of the discussion presented in a paper on "The Amphoteric Properties of some Amino-Acids and Peptides"[18] will be quoted.

"These substances (amphoteric electrolytes), depending upon conditions, ionize or react as salts in which the complex ampholyte component acts as the positive constituent or the negative constituent. . . . In order to account for the different processes of ionization, it appears to be necessary to assume that the molecule (unionized) is different in the two cases. . . . An equilibrium relation between the two (or more) unionized forms would exist as a rule. This conception complicates somewhat the theoretical treatment of amphoteric electrolytes presented by Bredig,[19] Walker,[20] and others by the addition of an equation representing such an equilibrium. . . .

"The difference in the ionization of the ampholyte is not always brought out clearly. For example, with a protein, the two kinds of ions are sometimes indicated as Na^+ (Protein$^-$) and (Protein$^+$) Cl^-. As a matter of fact, rarely, if ever, do the ions (Protein$^-$) and (Protein$^+$) have even the same chemical composition. This may be indicated by taking the simple case of glycine. Here the ions are $Cl^-(NH_3CH_2CO_2H)^+$ or $Cl^-(C_2H_6O_2N)^+$ and $(NH_2CH_2CO_2)^-Na^+$ or $(C_2H_4O_2N)^-Na^+$, or $(HONH_3CH_2CO_2)^-Na^+$ or $(C_2H_6O_3N)^-Na^+$, omitting possible hydration of the ions.

". . . The definitions of isoelectric points given by various workers have been contradictory at times. The most satisfactory definition appears to be that hydrogen ion concentration at which the properties such as electrical conductivity, viscosity, solubility, etc., when studied over extended ranges of acidity in moderately dilute solutions, show a point of inflexion. Chemically this would be interpreted as stating that in more acid solutions the substance ionizes with the complex as part of the cation, in more alkaline solutions, as part of the anion. At the isoelectric point combination with added acid or base and accompanying ionization is a minimum, or the substance is in a maximum uncombined state. . . .

[18] H. Eckweiler, H. M. Noyes, and K. G. Falk, *J. Gen. Physiol.*, *3*, 303 (1921).
[19] G. Bredig, *Z. Elektrochem.*, *6*, 33 (1899-1900).
[20] J. Walker, *Proc. Roy. Soc. London* (*B*) *73*, 155 (1904); *74*, 271 (1905); *Z. physik. Chem.*, *49*, 82 (1904); *51*, 706 (1905).

"The equation for calculating the isoelectric point of an amphoteric electrolyte

$$I = \sqrt{\frac{k_a}{k_b} k_w}$$

was deduced by Michaelis and Mostynski.[21] Their deduction does not appear to be altogether clear in so far as the assumptions involved are concerned. The following deduction brings out perhaps more satisfactorily these assumptions. It includes the view that the concentration of the unionized molecule in each case is given by the total number of molecules, not separate molecular species as indicated above.

$$k_a(HAOH) = (H^+)\ (AOH^-)$$
$$k_b(HAOH) = (OH^-)\ (HA^+)$$
$$\frac{k_a}{k_b} = \frac{(H^+)\ (AOH^-)}{(OH^-)\ (HA^+)}$$
$$= \frac{(H^+)^2}{k_w} \cdot \frac{(AOH^-)}{(HA^+)}$$

If $(AOH^-) = (HA^+)$

$$(H^+) = \sqrt{\frac{k_a}{k_b} k_w} \qquad (1)$$

This derivation assumes the equalities of (HAOH) in the two ionization equations and of (AOH^-) and (HA^+). In the Michaelis and Mostynski original deduction, the assumptions were stated to be the equality of the complex anion and cation concentrations and a minimum total ionization of the ampholyte.

"The following deduction involves the view of different molecular species ionizing as acid and as basic salts.

$$k_a(HAOH) = (H^+)\ (AOH^-)$$
$$k_b(HA_1OH) = (OH^-)\ (HA_1^+)$$
$$\frac{k_a}{k_b} = \frac{(H^+)\ (AOH^-)\ (HA_1OH)}{(OH^-)\ (HA_1^+)\ (HAOH)}$$
$$= \frac{(H^+)^2\ (AOH^-)\ (HA_1OH)}{k_w\ (HA_1^+)\ (HAOH)}$$

$$(H^+) = \sqrt{\frac{k_a}{k_b} k_w} \cdot \frac{(HA_1^+)}{(HA_1OH)} \cdot \frac{(HAOH)}{(AOH^-)} \qquad (2)$$
$$\text{(a)} \qquad \text{(b)} \qquad \text{(c)}$$

[21] L. Michaelis and B. Mostynski, *Biochem., Z.*, **24**, 79 (1910).

Equation (2) differs from equation (1) in containing the additional terms (b) and (c). For the two equations to give the same isoelectric point in any given case either of the following relations must hold (these are fundamentally the same with the terms arranged differently).

$$\frac{(HA^+)}{(AOH^-)} = \frac{(HA_1OH)}{(HAOH)} \tag{3}$$

$$\frac{(HA_1^+)}{(HA_1OH)+(HA_1^+)} = \frac{(AOH^-)}{(HAOH)+(AOH^-)} \tag{4}$$

. . . equations (1) and (2) are identical if $(HA_1^+)=(AOH^-)$ and $(HA_1OH)=(HAOH)$, the assumptions under which equation (1) was deduced.

"Equations (1) and (2) do not give the same isoelectric point, if the reciprocal of (c) is larger than (b), or the ionization as acid is greater than as base. The product of (b) and (c) will then be less than unity and the value obtained by means of equation (2) will be less than that given by equation (1). The reverse relation holds similarly.

"Because of insufficient data, the application of these relations is possible only in isolated cases. For simple substances, including amino-acids, etc., the isoelectric point as defined is probably identical with the hydrogen ion concentration of the pure substance dissolved in water. For more complex substances such as proteins, with a number of different acid- and base-combining groups, there will probably ordinarily be an overlapping of actions. The isoelectric point will then be the hydrogen ion concentration which involves a minimum combination with added acid or alkali, where the protein exists most nearly uncombined. The isoelectric point of a substance obviously shows the relative strengths of the substance acting as an acid and as a base.

"Equation (1) requires that the isoelectric point of an ampholyte does not change with change in concentration.[22] Some results with glycine and asparagine show definite if small changes in the hydrogen ion concentrations of solutions of these substances on dilution.[23] The use of equation (2) may help to explain these variations.

[22] Cf. H. T. Tizard, *J. Chem. Soc.*, *97*, 2490 (1910).

[23] Quoted by W. M. Clark, "The Determination of Hydrogen Ions," Second Edition, Baltimore, 1922, p. 39, from results of S. P. L. Sörensen.

"The agreement between the isoelectric points calculated by means of equation (1) and those found experimentally is surprisingly close in many cases. For substances such as glycine, etc., where the values of the acid and basic dissociation constants are not far removed from each other and the isoelectric points in the neighborhood of the hydrogen ion concentration of the solvent, this is not unexpected. For a substance like aspartic acid[24] for which $k_a = 1.5 \times 10^{-4}$ and $k_b = 1.2 \times 10^{-12}$, the calculated isoelectric point according to equation (1) is very nearly $(H') = 10^{-3}$ N. The value found by the indicator method was $(H') = 10^{-2.9}$ N. This can only mean that there is some sort of compensation with terms (b) and (c) of equation (2) resulting in the calculated values differing to only minor extents from those given by equation (1). It must be remembered, however, that this compensation is not a necessary conclusion in every case as far as known at present, but that differences may be shown by the two equations."

The principles developed in the study of amphoteric substances of comparatively simple composition should be useful in the interpretation of the chemical nature of all such substances. It would appear that amphoteric compounds are more widespread than has heretofore been assumed. In general terms such compounds include substances which in acid solutions show properties indicating electropositive characteristics, in alkaline solutions, electronegative characteristics, with an intermediate point, zone, or transition state which is considered to be either different from the other two states, or taken to be made up of the two states in definite proportions. For many compounds this transition state lies so far over in the acid or alkaline region that for most purposes it does not come into question. With many substances occurring in living matter, however, especially those of protein nature, the transition state, or isoelectric point, is not far removed from the ordinary conditions of acidity or alkalinity of the naturally occurring liquids or solutions. The amphoteric properties of these substances therefore assume special importance in the study of such substances, where comparatively small changes in hydrogen ion concentration may produce marked changes in the properties and reactions of the substances involved. It is not surprising, therefore, that much of the work on amphoteric

[24] K. Winkelblech, *Z. physik. Chem.*, *36*, 546 (1901); H. Lundén, *Z. physik. Chem.* *54*, 532 (1906); *J. Biol. Chem.*, *4*, 287 (1908).

substances has been done with proteins and other substances obtained from living matter, even if their chemical compositions and structures introduce apparently unnecessary and undesirable complexities.

The view of the writer with reference to these compounds is that the chemical properties, either of the constituent atoms or groups of atoms, will suffice to account for the properties which have been found. The earlier work of Sörensen and of L. J. Henderson and their co-workers furnishes important and valuable evidence in these directions,[25] as well as the more recent work of Willstätter, some of which has just been described and that of H. v. Euler.

In referring the amphoteric and colloidal properties of substances to their chemical compositions and structures, it must not be supposed that the problem has been completely solved or all of the reactions satisfactorily explained. This is far from being the case. In fact, a number of chemists and others still prefer to consider adsorption phenomena as fundamentally physical in many cases. The adsorption of gases on solid surfaces is generally cited as evidence for this view. As a matter of fact, at the present time it is difficult to account for such a phenomenon as the adsorption of argon by carbon on a chemical basis.[26] A chemical explanation has been developed for such adsorption phenomena by the introduction of "secondary" and "partial" valence concepts. It would lead too far to enter into these relations here. To the writer, their significance as developed is not altogether clear or satisfactory in the doubtful cases where definite chemical combination is not shown.[27] It would appear that the new views of atomic and molecular structures, including on the one hand transfer of electrons and their rearrangements to produce definite structures in atoms and molecules, and on the other hand, arrangements of atoms in solids as shown by crystal structures, which may in turn be applicable in modified forms to noncrystalline solids and to certain liquids, might throw light on the nature of the forces involved.

For the present, and especially with the substances which are

[25] For example, cf. S. P. L. Sörensen and others, *Compt. rend. trav. Lab. Carlsberg, 1919,* Vol. 12; also L. J. Henderson, E. J. Cohn, P. H. Cathart, J. D. Wachman and W. O. Fenn, *J. Gen. Physiol., 1,* 459 (1919), for the results obtained with gluten.

[26] J. Dewar, *Proc. Roy. Soc. London, 74A,* 124 (1905).

[27] Cf. "Catalytic Action," pp. 142-151.

more directly involved in enzyme and related actions, the chemical properties appear to be sufficient to account for the amphoteric properties observed and the adsorption reactions of the colloidal materials described. The adsorption compounds which are formed in many cases are not what is termed generally in a crude empirical way, very stable. Under certain conditions they dissociate readily into their original constituents. They do not dissociate under all conditions but only when treated with definite reagents. This was shown clearly by Willstätter in the elution of enzymes from their adsorption compounds. More or less specificity was found here in the decomposition of the adsorption compounds which permitted of a separation to a greater or less extent of the active enzyme material from inactive accompanying matter. This relative stability and specificity of action can be taken, of course, as evidence of the chemical nature of the reactions.

The adsorption compounds spoken of, do not show definite stoichiometrical relations except in isolated cases. This is not difficult to understand because of the difficulty of obtaining uniform mixture with the colloidal solutions which are involved. The particles present consist as a rule of "micelles," aggregates of molecules in the ordinary sense. Questions of penetration of surface of the reacting constituent; of membrane equilibria in which the concentrations of substances within the micelle in comparison with those in the solution without may, and frequently are, different; of reaction occurring at the surface without penetration of the micelle; etc., may be involved. The problem is more complicated than that of adsorption of a gas or material from solution by a metal, because membrane potentials and diffusions are involved. At the same time, a definite beginning has been made in their solution by the studies of various chemists, especially J. Loeb. The recent work of Northrop [28] with reference to the penetration of gelatin micelles by trypsin which was referred to before and which will be referred to again in later chapters, indicates the progress which is being made in the scientific study and interpretation of these phenomena.

Some phenomena which have been observed with enzymes will now be described which are connected with the colloidal or similar properties just taken up. These phenomena taken by themselves illustrate a common behavior difficult of explanation some years

[28] J. H. Northrop, *J. Gen. Physiol.*, *6*, 337 (1923-1924).

ago, but at the present time finding a ready place in the general scheme of enzyme actions and reactions because of some recent work especially by Willstätter and Euler.

The first interesting result is that described by Nelson and Griffin.[29] They found that a partially purified yeast sucrase preparation was not affected in its activity whether or not the enzyme was adsorbed by a solid like charcoal, or by a colloid like saponin, serum albumin, or egg albumin, or distributed uniformly throughout the solution of the substrate. Also, it was shown that the adsorbed sucrase could be displaced by a second colloid without any effect on its activity, and that it could be removed from an aqueous solution by adsorption by a solid and again brought into solution by displacement by a second colloid suspended uniformly throughout the solution. These early observations were developed and extended by Willstätter as already described. They are quoted here because they form the foundation for the quantitative study of yeast sucrase which was carried out by Willstätter. It was shown that the amount of sucrase present, as shown by its action, was constant, as a rule, from the beginning of the study of the original material, the yeast, to the highest state of purity obtained. The presence of foreign matter did not apparently influence its action in any of the tests. Because of this, it was possible to follow accurately the increase in purity or enzyme content of the material at various stages of the treatments.

The second series of experiments which led to a similar conclusion were carried out by Northrop [30] in working with pepsin. Northrop found that the state of aggregation of the protein, whether in solution or not, and the viscosity of the medium, exerted no marked influence on the rate of digestion of the protein.

The third result to be given includes some work with a castor bean lipase preparation [31] where it was found that "whether the material was dissolved in salt solution or suspended in the aqueous solution appeared to be of small influence on its hydrolyzing action."

These investigations, dealing with three such different enzymes as sucrase, pepsin, and lipase, although it is true that all exert hydrolyzing action on their individual substrates, show what might be

[29] J. M. Nelson and E. G. Griffin, *Jour. Amer. Chem. Soc.*, *38*, 1109 (1916).

[30] J. H. Northrop, *J. Gen. Physiol.*, *1*, 607 (1919); W. E. Ringer, *Z. physiol. Chem.*, *95*, 195 (1915), obtained similar results for the action of pepsin on edestin.

[31] K. G. Falk, *Jour. Amer. Chem. Soc.*, *37*, 226 (1915).

considered to be a more or less general physical property in that the state of aggregation apparently exerted little or no influence upon the action of the enzyme. It will be shown in the following chapter that this statement as representing a general truth is incomplete, that the nature of the adsorbing material with which the enzyme is precipitated or combined determines whether or not the enzyme shows its activity or can be made to recover it, and that any treatment with apparently inert material may result in either reversible or irreversible inactivation of an enzyme, depending upon the conditions employed, and the specific enzyme studied.

In the first edition of this monograph, the actions of enzymes at different hydrogen ion concentrations and the conditions for optimum actions were included in the description of the physical properties of enzyme preparations. They will be included here in the following chapter where some of the common chemical properties are described. It is entirely a matter of personal preference under which heading they should best be considered. The hydrogen ion concentration determines the amount of action of a given enzyme preparation, but the way in which it does this or the nature of the chemical changes which accompany change in hydrogen ion concentration are not known at the present time. Because of certain analogies to other chemical changes and deductions based upon plausible hypotheses, certain theories have been advanced which may well turn out to be correct and which will be indicated briefly in the following chapter.

V.—Chemical Properties Common to Enzyme *Preparations*

In taking up chemical properties common to enzyme preparations, the difficulty which is met with right at the start is the fact that at the present time enzymes are not known as chemical individuals or definite entities. Any discussion of the chemical properties of enzyme preparations would involve, therefore, the necessarily uncertain factor that the property under discussion may have no connection with the enzyme action. This necessary limitation in the treatment must be borne in mind, and while it introduces a doubtful element into the conclusions, it will be shown that the common chemical properties which will be discussed are of general enough relevance to aid, at the very least, in indicating the directions which further studies may take, and possibly also give results of immediate value. Some of the results which have been obtained for individual enzymes will be presented in the next chapter. In this chapter, only those relations which appear to have more or less general applicability to enzyme actions and reactions will be given.

In the experimental study of the chemical reactions whose velocities are influenced by enzymes, one of the striking facts to be first observed is that these influences are modified by many comparatively simple changes in the medium in which the reaction is occurring. Such changes have been studied rather extensively for the different enzyme materials. They may include the actions of acids, of alkalies, of neutral salts, of alcohols and other organic materials, of change in temperature, etc. Changes in enzyme and substrate concentrations as they may effect the kinetics of the actions will be taken up in a subsequent chapter. A systematic survey of the field is difficult because of the many variables involved in studying even one enzyme action. It will be attempted here, without, however, entering into the details of all the reactions which have been described, or even referring to studies which may appear relevant.

The first results to be given in discussing the chemical properties common to enzyme preparations involve the increases and decreases of the actions brought about by the changes in the hydrogen ion concentrations of the media. Since Sörensen [1] in 1909 first emphasized the importance of this factor in the quantitative study of enzymes, much work has been done in determining the actions of a large number of enzyme materials at different hydrogen ion concentrations. Every enzyme preparation shows a zone of greater or less extent for optimum action, the action being smaller in more acid or more alkaline solution. A number of these optima, expressed in terms of pH values, are shown for various enzymes in the following table:

	pH
Sucrase, yeast [1]	3.7–5.2
Sucrase, yeast [2, 3]	4.4–4.6
Sucrase, yeast [4]	4.63
Sucrase, potato [5]	4.–5.
Sucrase, banana [6]	4.0
Sucrase, intestinal [7]	6.8
Sucrase, pneumococcus [8]	7.
Sucrase, leucocytes [9]	7.9
Raffinase, yeast [10]	5.
Maltase, yeast [11]	6.6
Maltase, takadiastase (47°) [12]	7.2
Maltase, takadiastase (35.5°) [12]	3.
Emulsin (amygdalin) [13]	7.
Amylase, malt [14]	4.4
Amylase, takadiastase [14]	4.8
Amylase, saliva [15 *, 15a]	6.
Amylase, saliva [16]	6.4
Amylase, potato [5 *]	6. –7.
Amylase, cabbage, carrot, white turnip [17 *]	6.
Amylase, pancreatic [14]	7.
Amylase, yellow turnip [17]	4. –7.
Amylase, pancreatic [18]	6.8

* These results refer to saccharogenic actions.

[1] S. P. L. Sörensen, *Biochem. Z.* **21**, 131 (1909).

	pH
Amylase, liver [18a]	6.9
Amylase, phascolus vulgaris [19]	5.0–5.4
Urease, soy bean [20, 21]	7.0
Urease, soy bean [22]	7.3–7.5
Urease, (robinia) [23]	7.4
Pepsin (edestin, casein) [24]	1.4
Pepsin (egg albumin) [25]	1.4
Pepsin (caseinogen, 10-15 min.) [26]	1.8
Pepsin (egg albumin, ½-1 hr.) [2]	1.6
Pepsin (egg albumin, 12 hrs.) [2]	1.2
Pepsin, yeast (proteins) [27]	4.0–4.5
Pepsin, animal tissues (gelatin) [28]	3.0–3.5
Pepsin, animal (ovalbumin) [29]	1.5
Pepsin, animal [30]	1.5
Pepsin, yeast [30]	4.0–4.5
Pepsin, malt germ [30]	3.7–4.3
Pepsin, green malt [30]	3.2
Trypsin, pancreatic (albumose) [31]	7.7
Trypsin, pancreatic (casein) [32]	8.3
Trypsin, yeast (peptone) [27]	7.0
Trypsin, animal tissues (peptone) [28]	7.8
Trypsin, pancreatic [30]	8.0
Trypsin, yeast [30]	7.0
Trypsin, malt germ [30]	6.3
Trypsin, green malt [30]	6.3
Erepsin, intestinal (albumose) [33]	7.7
Erepsin, yeast (peptides) [27]	7.8
Erepsin, animal tissues (glycylglycine) [28]	7.8
Papain (egg albumin, gelatin) [34]	5.0
Protease, takadiastase (albumose) [35]	5.1
Protease, animal kidney (peptone) [36]	7.8
Protease, animal tissues (casein, peptone) [37]	7.0–7.5
Oxidase, vegetables [17]	7. –10
Peroxidase, vegetables [17]	7. –10
Catalase, vegetables [17]	7. –10
Catalase [2]	7.

	pH
Catalase, liver [31]	7.
Catalase [38, 39]	7.
Tyrosinase (bacterial) [10]	8.

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[15a] W. E. Ringer and H. van Trigt, *Z. physiol. Chem. 82*, 184 (1912).
[16] U. Olsson, *Z. physiol. Chem. 117*, 91 (1921).
[17] K. G. Falk, G. McGuire, and E. Blount, *J. Biol. Chem. 38*, 229 (1919).
[18] R. Willstätter, E. Waldschmidt-Leitz and A. R. F. Hesse, *Z. physiol. Chem. 126*, 143 (1923).
[18a] O. Holmbergh, *Z. physiol. Chem. 134*, 68 (1924).
[19] K. Sjöberg, *Biochem. Z. 133*, 218, 294 (1922).
[20] E. K. Marshall, Jr., *J. Biol. Chem. 17*, 351 (1914).
[21] D. D. van Slyke and G. Zacharias, *J. Biol. Chem. 19*, 181 (1914).
[22] P. Rona and G. György, *Biochem. Z. 111*, 115 (1920).
[23] S. Nakagawa, *Mitt. med. Fak. Kais. Univ. Tokyo, 28*, 383 (1922).
[24] L. Michaelis and A. Mendelssohn, *Biochem. Z. 65*, 1 (1914).
[25] S. Okada, *Biochem. J.* 10, 126 (1916).
[26] L. Michaelis and H. Davidsohn, *Z. exp. Path. Therap. 8*, 398 (1910).
[27] K. G. Dernby, *Biochem. Z. 81*, 109 (1917).
[28] K. G. Dernby, *J. Biol. Chem. 35*, 179 (1918).
[29] Shin Shima, *J. Biochem. (Japan) 2*, 207 (1923).
[30] H. Lundén, *Biochem. Z. 131*, 193 (1922).
[31] L. Michaelis and H. Davidsohn, *Biochem. Z. 36*, 280 (1911).
[32] H. C. Sherman and D. E. Neun, *Jour. Amer. Chem. Soc. 38*, 2203 (1916); *40*, 1138 (1918).
[33] P. Rona and F. Arnheim, *Biochem. Z. 57*, 84 (1913).
[34] E. M. Frankel, *J. Biol. Chem. 31*, 201 (1917).
[35] S. Okada, *Biochem. J. 10*, 130 (1916).
[36] S. G. Hedin, *Z. physiol. Chem. 122*, 307 (1922).
[37] K. G. Falk, H. M. Noyes, and K. Sugiura, *J. Biol. Chem. 53*, 75 (1922).
[38] S. Morgulis, *J. Biol. Chem. 47*, 34 (1921).
[39] P. Rona and A. Dambovíceanu, *Biochem. Z. 134*, 20 (1922).
[40] C. Stapp, *Biochem. Z. 141*, 42 (1923).

The results given in the table are not intended to show all the determinations of conditions of optimum hydrogen ion concentrations for enzyme actions which are recorded in the literature. Enough are given to illustrate the nature of the results and to make possible a study of the general relations involved.

The first point to be taken up in the consideration of these results is indicated clearly by a quotation taken from one of Sörensen's earliest papers on the significance of the hydrogen ion concentration in enzyme studies. As a conclusion drawn from the work of O'Sullivan and Tompson [2] on sucrase and from some of his own work he stated: [3] "An interrelation exists between the three factors: temperature, hydrogen ion concentration, and time. Optimum hydrogen ion concentration of an enzyme action varies, within fairly narrow limits, with the temperature and time of the experiment, and even the optimum temperature of such a reaction without doubt will vary with the time and the hydrogen ion concentration of the mixture, probably within narrow limits, depending upon the magnitude of the temperature coefficient of the velocity of inactivation of the enzyme. In indicating the optimum temperature and hydrogen ion concentration, the experimental conditions should also be given."

The hydrogen ion concentration is, then, one of the factors upon which an enzyme action depends. It is the most important single factor in many cases, but for some enzyme actions other factors play just as important a part. It is an invaluable property in connection with the study of an enzyme action obtained from different materials and for the quantitative study of comparative actions.

That an enzyme, depending upon its source, may have different optimum conditions even when acting on the same substance is shown by the results with sucrase and amylase. The yeast and vegetable sucrases show optimum actions at pH 4 — 5, the intestinal and other sucrases in more alkaline solution. The action of the yeast sucrase is not influenced apparently by the presence of impurities as far as the optimum pH conditions are concerned. It must be noted, however, that the intestinal sucrase has not as yet been obtained in solution, the studies with it having been carried out with the enzyme material in suspension. Similar relations were

[2] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc.* 57, 834 (1890).
[3] S. P. L. Sörensen, *Biochem. Z.* 21, 131 (1909).

found with the amylases, striking differences for the pH optima being found for the materials from different sources.

Paralleling these relations are the different optima found for the same enzyme material when acting upon different substrates, as found for pepsin, for example. Part of these differences, at least, are accounted for by the ionization of the enzyme material and of the substrate, as shown by Northrop.[4] Definite ionization relations must be realized for optimum actions.

Following these relations, it is not surprising to find that a series of enzyme preparations, taken to be similar at first sight, such as the proteases, but found to act differently upon different substrates, are made up of entirely different enzymes; for the protease actions, of pepsins, trypsins, and erepsins. This classification is based upon the source of the enzyme material, the substrate upon which it acts, and the optimum conditions for the actions. It is probable that some of the confusing relations which have been observed with certain enzyme actions will be explainable as due to several enzymes of the same general type, acting simultaneously.

Several additional factors must be taken up in connection with the optimum pH conditions for actions, especially with enzymes which are markedly unstable. Some results obtained with the ester-hydrolyzing enzymes (or lipases) of animal tissues may be given as examples.[5] The influence of the hydrogen ion concentration on lipase was studied in two ways. In the first place, the aqueous extracts of the tissues were brought to different hydrogen ion concentrations with sodium hydroxide or hydrochloric acid, ester added, and the amounts of hydrolysis measured by titration after a suitable time interval. In the second place, portions of the extracts, after being brought to different hydrogen ion concentrations, were allowed to stand for from 12 to 24 hours, then all brought back to the same state, pH 7.0, ester added, and the amounts of hydrolysis measured after a suitable time interval.

The two methods do not determine the same property. The first method measures the amount of hydrolysis produced by the lipase material at different hydrogen ion concentrations. The second method measures the amounts of inactivation of the lipase material

[4] J. H. Northrop, *J. Gen. Physiol. 3*, 211 (1920-21).
[5] H. M. Noyes, K. Sugiura, and K. G. Falk, *J. Biol. Chem. 55*, 653 (1923).

at different hydrogen ion concentrations by means of tests at the same hydrogen ion concentration.

As an added complication in studying these lipase actions, it may be pointed out that mixtures initially more alkaline than pH 5.0 tended to approach this condition of acidity as the enzyme action proceeded.

The results of the experiments showed two facts definitely. In the first place, the lipolytic actions of the tissue and tumor extracts showed no optimum at any hydrogen ion concentration tested, but increased in magnitude as the alkalinity of the mixtures increased. A careful consideration of the experimental results of others[6] indicates that fundamentally this behavior is borne out by their findings, although at times different conclusions may have been drawn. In the second place, the mixtures when tested at the same hydrogen ion concentration after being allowed to stand at different hydrogen ion concentrations, showed the smallest inactivations in the neighborhood of pH 6.5 to 7.5 in comparison with more acid and more alkaline solutions.

Two factors were apparently operating simultaneously in these experiments; enzyme action at a definite hydrogen ion concentration, and inactivation of enzyme at that hydrogen ion concentration. The time and temperature factors are also involved, especially in the inactivations.

These considerations apply to a number of enzyme actions. Their interpretation is most readily shown in the experiments on lipase quoted, but obviously are of general applicability. A reason is evident, therefore, for different observers obtaining different pH optima for an enzyme action where the same pH may have been expected. The experimental conditions must be carefully scrutinized in every case. This is true even when an added substance, such as perhaps a buffer mixture, is present. The relations with added substances will be taken up presently.

In place of continuing the discussion of the behavior of enzymes as such, a digression will be made in order to compare the actions of hydrogen and hydroxyl ions on certain chemical reactions alone and in the presence of enzymes.

[6] H. Davidsohn, *Biochem. Z.* *45*, 284 (1912); *49*, 249 (1913). P. Rona and Z. Bien, *Biochem. Z.* *59*, 100 (1914); *64*, 13 (1914). O. T. Avery and G. E. Cullen, *J. Exp. Med.* *32*, 57 (1920). F. A. Stevens and R. West, *J. Exp. Med.* *35*, 823 (1922).

The hydrolysis of sucrose by acids, as shown in an earlier chapter, increases with increase in hydrogen ion concentration, not strictly quantitatively except for limited ranges. The rate of change is negligible at moderate hydrogen ion concentrations ($10^{-5.0}$ N or less). The catalytic action is only observed, therefore, in solutions which are ordinarily considered to be acid. The action of the enzyme sucrase at different hydrogen ion concentrations is in marked contrast to these actions. As shown in the table, a number of the sucrase preparations show optima at pH 4.5, being inactive in the neighborhoods of pH 2.5 and 7.5, while the pneumococcus sucrase had its optimum at pH 7.0, and the intestinal sucrase at pH 6.8.

The work of Northrop, which was also described in Chapter II, on the hydrolysis of proteins by acid and alkali, showed increasing actions in both acid and alkaline solutions with a minimum at about pH 6.0. The protease actions which are given in the table show optimum actions at pH values which cannot be considered to be related to the type of action found without the enzyme. Similarly with the hydrolysis of esters where a minimum is found at pH 6.0 in the absence of enzymes, but in their presence, entirely different relations are apparent.

The relation between an enzyme action and the hydrogen ion concentration is generally represented by a pH-activity curve. These curves have been taken to represent various relations such as with sucrase the dissociation curve of an enzyme substance[7] or more successfully of an enzyme-substrate compound,[8] the enzyme or enzyme-substrate acting as a weak base or acid and forming ionizable salts with the acids or bases present. These explanations account for a certain number of the facts observed, and have been found useful in correlating various relations.

It was shown that the chemical reactions which are catalyzed by enzymes over limited ranges of acidity, are catalyzed by acids or alkalies over markedly different ranges. These enzyme actions must be ascribed to factors not included in the hydrogen or hydroxyl ions as such.

It may appear as if these limited ranges of acidity in connection with certain chemical properties which have been found useful for

[7] L. Michaelis and M. Menten, *Biochem. Z. 49*, 333 (1913).

[8] L. Michaelis and M. Rothstein, *Biochem. Z. 110*, 217 (1920); H. v. Euler, K. Josephson, and K. Myrbäck, *Z. physiol. Chem. 134*, 39 (1924).

certain purposes are unique. This is by no means the case, and the manner in which the preceding statement was made indicates at once another group of substances which possess certain chemical and physical properties of value to chemists over more or less limited ranges of hydrogen ion concentrations. These are the indicators. A brief outline of the theories which were developed for indicators and their uses may be of interest here, as a surprising parallelism to the present development of enzyme theories is apparent.

Before the development of the electrolytic dissociation theory of Arrhenius, a satisfactory systematization of the color changes of indicators was not possible. W. Ostwald [9] in 1894 attributed the different color of an indicator in acid or alkaline solution to the different colors of the ions and the unionized molecules. If the indicator substance itself was an acid, the color in acid solution would be that of the unionized molecule, while in alkaline solution the color would be that of the negative ion. If the indicator substance was a base, the color in alkaline solution would be that of the unionized molecule, in acid solution that of the positive ion.

This theory was shown not to be general enough to include the observed phenomena, and was replaced by the "chemical" theory first suggested (for phenolphthalein) by Bernthsen, and developed by J. Stieglitz [10] who brought the chemical theory into harmony with Ostwald's theory of the sensitiveness of indicators, and by A. Hantzsch,[11] who showed the ionic theory of indicators to be highly improbable. The newer view considers every change in color of an organic substance to be due to an intramolecular rearrangement. Indicators form a special group in so far as the intramolecular rearrangements in their cases are tautomeric in character and include, therefore, in most cases the shifting of a hydrogen atom in passing from one form to the other. The production of ions is secondary in the tautomeric changes, and if the ions are colored, it is because the unionized molecules from which they are derived are colored. The equilibrium between the tautomeric forms of a substance depends upon a variety of factors such as solvent, temperature, small amounts of certain added substances such as acids and bases, etc. To illustrate this, a few results obtained with ethyl acetoacetate

[9] "Die wissenschaftlichen Grundlagen der analytischen Chemie," p. 104.

[10] *Jour. Amer. Chem. Soc.* **25**, 1112 (1903).

[11] *Ber.* **33**, 1084 (1906) and numerous articles since. Cf. also, among others, D. Vorländer, *Lieb. Ann.* **320**, 116 (1902); *Ber.* **36**, 1845 (1903).

may be quoted. The equilibrium between the tautomeric forms of this substance varies greatly in different solvents, the extreme values given by K. H. Meyer[12] being 0.4% enol form present in 3-5% aqueous solution at 0°, and 48% in hexane at 20°. A. Hantzsch[13] showed the important part played by solvents in affecting the equilibrium between the tautomeric forms of some indicators, and, therefore, the color changes of indicators. The action of acid and of alkali on the equilibrium between tautomeric forms is well known. Similar actions take place with indicators in aqueous solution, one form predominating in the presence of acids; the other (tautomer) in the presence of bases. In practical titrations, the indicator substance is present in such small concentration that the color change which accompanies the transformation of one tautomer into the other is very marked with the relatively small amount of added substance necessary to produce it.[14] Other changes of conditions may be considered similarly for the indicators as a special class of tautomeric substances.[15] In general, it may be stated that the various factors which influence the equilibrium between tautomers also influence the equilibrium between the different tautomeric forms of indicators, and that the question of the electrolytic dissociation of the indicator substances does not enter into the theory of their color changes as assumed in the earlier theory, although it appears to be connected with one of the factors involving the sensitiveness. Some years ago, W. Ostwald[16] suggested the view that the colors of indicator substances were dependent upon their degrees of dispersion, and that acids and bases brought about color changes with them simply by altering the dispersion. This theory, as an explanation of the phenomenon has not found favor, however, and need not be considered further at present.

If, in place of color change of indicators at a certain hydrogen ion concentration, chemical change due to enzyme is substituted, a number of striking similarities are apparent. Usefulness over a limited range of acidity may mean in terms of chemical configuration, that a certain definite chemical structure or relationship or combination between the atoms is present over that range and is

[12] *Ber. 45,* 2843 (1912).
[13] *Z. Elektrochem. 20,* 480 (1914); *Ber. 48,* 158 (1915).
[14] In this connection cf. A. A. Noyes, *Jour. Amer. Chem. Soc., 32,* 815 (1910).
[15] For the action of neutral salts, cf. L. Rosenstein, *Jour. Amer. Chem. Soc. 34,* 1117 (1912).
[16] *Z. Chem. Ind. Kolloide 10,* 132 (1912); cf. also G. Wiegner, *Mitt. Lebensm. Hyg. 11,* 216 (1920).

modified or changed at different acidities. These changes are in the main reversible with indicators, very often irreversible with enzymes. Added substances, such as salts, etc., may modify the changes to small extents in some cases, to large extents in others. The development of the theories to account for the color changes are also similar to the present developments with enzymes; views involving colloidal properties, hydrogen ions, and finally the chemical theory, which has now been widely accepted and which has as its basis chemical structure as the reason for the definite property.

The question of change in activity of enzyme preparations by added substances has been studied extensively. Such substances may either increase the actions or decrease them. If they do not change the velocities of the actions it is naturally assumed that they do not act upon the enzyme, but this conclusion does not follow necessarily.[17]

The first action which may be considered is the possible influence of substances added to the mixtures to bring them to the desired hydrogen ion concentration. If acid or base is added, presumably there is no such influence, if buffer mixtures are used, there may be such effects. With yeast sucrase, for example, and small amounts of buffer mixtures, no apparent influence is observed due to the nature of the salt mixture used. With banana sucrase, however, citrate buffer mixtures show a retarding influence increasing with their concentrations, especially in the more alkaline solutions, phosphate buffer mixtures greater retarding actions, and phthalate and acetate mixtures still greater.[18]

Similar results have been obtained with saliva amylase. Different observers obtained at times somewhat different values for the hydrogen ion concentration for optimum action, depending upon the buffer mixture used and also in some cases upon the neutral salt present. Thus, W. E. Ringer and H. van Trigt [19] found for optimum action a pH of 6.0 in the presence of phosphate or acetate, while with citrate the value was influenced by the concentration of the buffer; L. Michaelis and H. Pechstein [20] found a pH 6.1-6.2 with phosphate, arsenate, and sulfate, pH 6.7 with chloride and bromide, and pH 6.9

[17] Cf. K. G. Falk, "Catalytic Action," p. 31.
[18] G. McGuire and K. G. Falk, *Jour. Amer. Chem. Soc.* *45*, 1539 (1923).
[19] *Z. physiol. Chem.* *82*, 484 (1912).
[20] *Biochem. Z.* *59*, 77 (1914).

with nitrate; and A. Hahn and R. Michalik [21] found pH 6.6 with phosphate mixture and pH 5.6 with acetate mixture.

The point illustrated with the sucrase and saliva amylase is of extreme importance in connection with the study of the hydrogen ion concentration for optimum actions in so far as the use of buffer mixtures is concerned, and also in the study of the actions of salts on enzymes at definite hydrogen ion concentrations. For example, to refer to yeast sucrase again, Fales and Nelson [22] found that at the optimum hydrogen ion concentration for its action, the addition of sodium chloride had practically no effect on the velocity of the hydrolysis of sucrose. At all other hydrogen ion concentrations, sodium chloride exerted an inhibiting action, greater progressively as the acidity or alkalinity was increased.

It has frequently been observed that inorganic salts influence enzyme actions. (It may be recalled that recent studies have shown that inorganic salts may influence the color changes of indicators, and that different indicators may act differently.) With any one enzyme, addition of a salt may increase or diminish the velocity of the enzyme action. It is impossible to predict what the action will be, but on the other hand, the actions are sometimes so striking that they would appear to offer the most direct clue to the chemical nature of the enzyme. For example, the activating action of cyanide on the proteolytic enzyme papain is very large.[23] Also, the activating effect of bromide on amylase is great, differing in this respect from chlorides and iodides.[24] The action of manganous sulfate on castor beans, activating the lipase, may also be referred to.[25]

The hydrogen ion concentration has not been carefully controlled in many salt studies, and although this introduces an element of doubt, still some interesting conclusions are possible. For example, the results of the study of the actions of a number of neutral salts on the action of a castor bean lipase preparation toward ethyl butyrate [26] may be quoted, especially since here, the hydrogen ion concentration is not the main factor governing the activity, but only

[21] *Z. Biol. 73,* 10 (1921).
[22] H. A. Fales and J. M. Nelson, *Jour. Amer. Chem. Soc. 37,* 2769 (1915).
[23] E. M. Frankel, *J. Biol. Chem. 31,* 201 (1917).
[24] A. W. Thomas, *Jour. Amer. Chem. Soc. 39,* 1501 (1917).
[25] E. Hoyer, *Z. physiol. Chem. 50,* 414 (1907); Y. Tanaka, *Orig. Com. 8th Intern. Congr. Appl. Chem. 11,* 37 (1912); K. G. Falk and M. L. Hamlin, *Jour. Amer. Chem. Soc. 35,* 210 (1913).
[26] K. G. Falk, *Jour. Amer. Chem. Soc. 35,* 601 (1913).

one of several factors, neutral salts exerting as great an influence. In every case the change in activity was found to be a continuous function of the concentration of the salt added. Decreased activities, as compared with the aqueous solutions, were shown by all the uni-univalent salts, by the chlorides and nitrates of barium and calcium (except for the most dilute solutions) and magnesium, by sodium oxalate, and by dilute solutions of sodium sulfate. Increased activities were shown by dilute solutions of the chlorides of barium and calcium, by more concentrated solutions of sodium sulfate, by magnesium sulfate, and by the chloride and sulfate of manganese. Potassium sulfate caused no change. It is possible, by a careful study of these results, to point out series of regularities. For instance, for the sodium and potassium halides, the retardations increased in the order chloride, bromide, iodide, fluoride. Terroine [27] had found the same order for the actions of the sodium halides on pancreatic lipase. The lithium salts exerted greater retarding actions than did the sodium and potassium salts. Such regularities might be multiplied, the positive and negative constituents of each salt, or possibly the ions, apparently exerting their individual actions in each case, which sum up to give the total action. A number of investigations of the actions of salts on enzymes might be quoted, all carried out in an analogous manner. A certain number of regularities might be deduced from each investigation, but unfortunately most of them suffer from the lack of exact control of the hydrogen ion concentrations of the mixtures. It may also be pointed out that the actions of salts on enzymes is reversible in some cases, the enzyme returning to its original activity upon the removal of the salt, and irreversible in others, the enzyme being permanently inactivated. The results can therefore be considered of value mainly from a qualitative point of view, and while unquestionably indicating certain facts of interest, at present do not appear to lend themselves to the development of the more exact chemical studies which are needed for a systematic following up of the enzyme problem. In the next chapter, some results more quantitative in character obtained with a highly purified sucrase preparation and possibly leading to more definite conclusions with regard to the probable chemical nature of this enzyme, will be presented.

It has been stated that serum albumin or charcoal or glass

[27] E. F. Terroine, *Biochem. Z.* **23**, 429 (1910).

beads inhibited the actions of certain enzymes such as sucrase.[28] Griffin and Nelson [29] showed that these inhibiting actions did not occur with sucrase if the hydrogen ion concentrations were kept unchanged, and that the actions of these added substances caused the retardations in the same measure that they changed the hydrogen ion concentration.

The interpretation of the action of salts is not easy. The treatments are in the main very simple. There seems to be little chance for any deep-seated chemical change to take place to bring about such marked activations or inactivations as are observed at times. That some change takes place is unquestionable. Again the similarity in the marked color changes of indicators brought about by simple treatments and the marked changes in chemical actions of enzyme preparations also brought about by simple treatment brings to mind the possibility of a similar underlying chemical transformation or change being responsible for both. This change would involve some intramolecular rearrangement. This question will be taken up in more detail in the next chapter.

The actions of certain neutral organic substances may also be taken up. The addition of alcohol precipitates most of the enzyme preparations from their solutions. Different concentrations are required for different enzymes, and it has been found possible to use a fractional precipitation at different concentrations to aid in separating a part of the accompanying inactive material. This is illustrated by the procedure developed by Sherman and his coworkers with amylases from different sources, and by others. The precipitates from the alcohol solutions can be dried with ether and studied further. It is found then that some of the enzyme preparations retain their activity while others lose it by this treatment. Sucrase, amylase, papain, and others are not affected within certain limits [30] as a result of the alcohol precipitation and subsequent drying, while esterase, lipase,[31] and maltase,[32] are completely inacti-

[28] E. Beard and W. Cramer, *Proc. Roy. Soc. London (B) 88,* 575 (1915); A. Erikson, *Z. physiol. Chem. 72,* 313 (1911).

[29] E. G. Griffin and J. M. Nelson, *Jour. Amer. Chem. Soc. 38,* 722 (1915).

[30] The concentration of the alcohol may have a marked effect. Thus, it is stated that with yeast sucrase there is a maximum inactivating action with 50% alcohol and none at all if the concentration of the alcohol is less than 20% or more than 80%. This result has, however, been questioned.

[31] K. G. Falk, *Jour. Amer. Chem. Soc. 35,* 616 (1913).

[32] W. A. Davis, *Biochem. J. 10,* 31, 57 (1915).

vated by the treatment. In all cases, as far as can be told, no physical or chemical change other than that indicated has taken place. The addition of acetone in place of alcohol produces the same result. The addition of certain salts parallels these actions. Treatment with sodium fluoride for example inactivates the enzymes esterase and lipase, at the same time precipitating them. Other salts have very slight actions or none, while others again increase the actions of the enzymes as already indicated. The reverse actions are also true in some cases. For example, the presence of a certain amount of inorganic salt, such as sodium chloride, is necessary in order to have amylase exert any action.[33] The removal of all of this salt inactivates the amylase, but addition again restores the activity. Various salts act differently; the apparently specific activations of certain of these which were given earlier in this chapter being especially interesting.

Some additional interesting results on lipase have been published recently.[34] The action of pancreatic lipase on glyceryl tributyrate was inhibited by quinine but not by atoxyl, while liver lipase was sensitive to atoxyl. Kidney lipase was not affected by quinine but was inhibited by atoxyl, but unlike liver lipase, blood serum did not protect the kidney lipase against atoxyl. The three lipases could therefore be differentiated in this way.

An extensive study[35] of the actions of various foreign bodies on the enzyme urease may also be referred to in this connection.

These relations appear to be very confusing. Perhaps they should be considered in connection with the adsorption experiments described in the preceding chapter. The first apparent similarity of change in enzyme action by the addition of other substances is connected with the precipitation or coagulation of the enzyme preparations. A closer study of the changes reveals the fact that such precipitation may occur with unchanged activity, and that apparently reversible precipitation or coagulation may be accompanied by no other change in physical or chemical property which has been followed experimentally except change in enzyme activity.

[33] Cf. L. Petri, *Biochem. Z.* *4*, 1 (1907); T. B. Osborne and G. F. Campbell, *Jour. Amer. Chem. Soc.* *18*, 536 (1896); H. C. Sherman and co-workers, *Ibid.*, series of papers, 1914-20.

[34] P. Rona and R. Pavolvic, *Biochem. Z.* *134*, 108 (1922); P. Rona and M. Takata, *Biochem. Z.* *134*, 118 (1922); P. Rona and H. E. Haas, *Biochem. Z.* *141*, 222 (1923).

[35] P. Rona and P. György, *Biochem. Z.* *111*, 115 (1920).

This leads at once to an interesting deduction. If it has not been possible, heretofore to detect experimentally any other chemical change where change in enzyme action occurs, it would follow that change in enzyme action affords one of the most sensitive, if not the most sensitive, criterion of change taking place in biological or biochemical material. No other chemical method at present appears to be capable of detecting the physical or chemical changes which take place when, under simple treatments, enzyme actions are modified. An experimental study of tissue enzymes based primarily upon these considerations will be presented in part in Chapter IX.

It may be thought that changes in enzyme material, such as have just been described, are due possibly to changes in surface, but again the writer prefers to consider the changes as fundamentally chemical in character, and the changes in surface, if such occur, to be secondary to, and dependent upon, the chemical changes.

It appears also that there is some connection between the colloidal properties of an enzyme preparation and the comparative stability of the enzyme. The colloidal properties are connected in a measure with a complex chemical structure or composition. That is to say, colloidal properties are found frequently with substances of large molecular weight. While, therefore, the colloidal property is found to be a characteristic of enzyme preparations, it is primarily evidence of the fact that such preparations are derived from biological material, and also may be connected, through the complexity of the molecule, with the possibility of retaining the enzyme activity in the molecule, or some particular part of the molecule, by the influence of the rest of the molecule and its complexity. Evidence for this may be seen in the fact, frequently observed, that separation of the enzyme preparation from inactive material accompanying it in the natural state, results in the enzyme becoming more sensitive to changes of inactivation. For example, O'Sullivan and Tompson [36] found that the conditions which resulted in the inactivation of a yeast sucrase solution at 50° in the absence of sucrose, in the presence of sucrose did not inactivate the sucrase at 60°, and inactivated it only partially at 70°. The fact that increase in purity of the yeast sucrase material decreased its stability was also

[36] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc.* 57, 834 (1890).

emphasized by Willstätter, Graser and Kuhn,[37] and by Nelson and Kerr.[38] Bayliss and Starling [39] showed that trypsin (obtained from pancreatic juice) was autolyzed much more slowly in the presence of proteins and peptones than in their absence. Osborne and Campbell [40] found that with amylase, the purer the preparation, the more sensitive was the enzyme to external conditions. The results of Sherman [41] point in the same direction. The following experiment of Bayliss [42] is also of interest in the connection. The presence of charcoal in a solution of trypsin preserved the enzyme to a considerable extent when heated to 60° for ten minutes; one-seventh less was destroyed than in the absence of charcoal, and under the conditions used charcoal was not a very effective adsorbent for trypsin.

A common characteristic of enzyme preparations is their inactivation by heating in aqueous solution. This property is sometimes used as one of the criteria as to whether a given reaction involves an enzyme action. There is no one temperature at which inactivation of all enzymes occurs, but the temperature, the time of heating, the presence of other substances, all influence the rate of inactivation. Unquestionably, such inactivations are due to chemical changes within the molecule, and other changes, such as coagulation, etc., are secondary.

This inactivation by heat of practically all enzymes also necessitates the conclusion that even at comparatively low temperatures gradual loss of activity occurs in solution. It is therefore advisable to keep enzyme solutions at low temperatures, for them to retain their activity as far as possible. No other chemical or physical change has been observed in many of these inactivations; in others, coagulation and precipitation occur; in still others, change in hydrogen ion concentration of the medium occurs.

As a rule, enzymes show their greatest catalytic actions at temperatures in the neighborhood of 40°. At higher temperatures it is probable that inactivation of the enzyme takes place with sufficient rapidity to cause apparent decrease in catalytic action.

[37] R. Willstätter, J. Graser and R. Kuhn, *Z. physiol. Chem. 123*, 1 (1922).
[38] J. M. Nelson and R. W. E. Kerr, *J. Biol. Chem. 59*, 495 (1924).
[39] W. M. Bayliss and E. H. Starling, *J. Physiol. 30*, 61 (1903).
[40] T. B. Osborne and G. F. Campbell, *Jour. Amer. Chem. Soc. 18*, 536 (1896).
[41] H. C. Sherman, *Jour. Amer. Chem. Soc.*, 1914-1920.
[42] W. M. Bayliss, *Proc. Roy. Soc. London (B) 84*, 81 (1911).

The question may now be considered whether it is possible to go further at present with the general property of chemical structure for such bodies as enzymes. Some space may be devoted to the fundamental properties of proteins and their simpler component parts and also of other biological materials, since the principles involved are apparently of general applicability.

For the moment, the discussion will be limited to the nitrogenous bodies. If an aminoacid is dissolved in water, the solution will possess a certain hydrogen ion concentration. This will vary to a certain extent with the concentration of the aminoacid as pointed out in the preceding chapter, but only to a minor extent (and for the purpose in view this is negligible) with most of them. This hydrogen ion concentration is the isoelectric point of the aminoacid at which it is combined to a minimum extent with either acid or base. Adding acid or base in definite amounts to this solution, determining the resulting hydrogen ion concentrations, and plotting the amounts of acid and alkali against the hydrogen ion concentrations in terms of pH will give the titration curve of the substance. Each substance, or possibly each group or type of substance, should have a more or less characteristic titration curve, depending upon the chemical groupings present. This is true as well for peptides, peptones, proteins, etc., as for aminoacids. Each substance would be expected to have a definite isoelectric point, and following the developments of the preceding chapter, the properties of the substance would change in passing from the acid side of the isoelectric point to the alkaline. This method of treatment and the development of the use of titration curves in the study of the properties of proteins was developed especially by J. Loeb, L. J. Henderson, E. J. Cohn, and their co-workers.

In the determination of the titration curves of proteins containing a number of potential basic and acidic groups, it is possible that in the addition of acid or alkali, combination with certain groupings takes place first and that in a sense there is a progressive neutralization of the basic or the acidic groups in the molecule. It is then conceivable that at some such point of partial neutralization, optimum conditions of enzyme action would be obtained, due either to the setting free chemically (possibly by rearrangement) of the active grouping, or a similar removal of an inhibiting grouping. This explanation is hypothetical, it is true, but it seems as if there must

be some such chemical reason to account for the observed facts. The changes in colloidal properties which may occur are to be referred back to the differences in the chemical groups due to the added acid or alkali.

The question whether tautomerism or desmotropism plays a part here in a manner analogous to its action in indicators can only be referred to and will be taken up again in the following chapter.

The discussion of these last questions has been limited to proteins. It is evident that other substances not protein in character but which may also be present in enzyme preparations might show the same relations. For example, the carbohydrate-phosphoric acid complexes which appear to be present in certain preparations, and possibly other phosphoric acid derivatives, might show these properties. In general, the same relations might be expected to hold and the influence of the hydrogen ion concentration taken to be fundamentally chemical in character and to modify the chemical state of the enzyme molecule or part of it.

In discussing titration curves and isoelectric points of proteins and other biological materials, it must be remembered that any chemical treatment to which the protein is submitted may change its properties. Thus, repeated solution and precipitation, whether by the action of acid and base, or by salt solution and dialysis, or by alcohol and acetone, will unquestionably modify the properties. As pointed out already, the enzyme property is one of the most easily modified of the properties of such materials, so that enzyme action may be destroyed even without experimental evidence of other changes. While much can be done in the study of purified or modified biochemical materials, in order to obtain evidence of the properties of the substances as they exist in living matter as nearly as possible, their properties and reactions must be followed before such changes have taken place.

In considering the occurrence of enzymes in living matter, it is evident, if enzymes play a part in the chemical changes which occur during growth and life processes, perhaps in a regulatory or directive manner, that the nature of these enzymes would be different at different periods of growth and life. This has frequently been observed, for example, in the more abundant occurrence of enzymes in germinating seeds, in the character of the sucrolytic enzymes present in yeast after causing it to live and reproduce in the presence and

by the utilization of certain saccharides,[43] in living animals in the secretion, and therefore production, of digestive juices containing enzymes upon the presentation of certain stimuli, etc. All these changes, and many more which might be listed, occur during the life processes of organisms. It is of interest to note that increases in activity of four different enzymes have been observed in materials apart from the life process and without addition of any reagent.

It was found[44] that the sucrase activity of banana extracts increased on standing for a certain period of time and then decreased again. The increases amounted to from 40 to 100% of the original activity, were independent of the composition of the extracting solutions and of the preservative used, were not due to the presence of banana cells or bacteria, and were not accounted for by changes in hydrogen ion concentration. The natures and amounts of increases were found, however, to be dependent upon the state of ripeness of the banana when extracted. A number of results with the sucrase of yeast[45] while not as clear-cut as those just presented with banana sucrase indicate similar changes. Willstätter and Pollinger[46] found that a number of peroxidase preparations (from turnips) spontaneously increased in activity, at times as much as 40-50%. These increases occurred both with crude and with purified preparations. In solution, they occurred in several days or less, in dry powdered form the increases were observed after standing months or even years. A number of tissue extracts upon standing at room temperatures for some weeks were found to have increased in some of their lipase or esterase activities.[47] Testing the actions upon a number of esters, greater action was observed upon some of them, less upon others, and no change upon still others. The increases amounted in some cases to 80%. Similar results, though less striking, were observed with some tissue proteases.

These spontaneous activations occurring with such different enzyme preparations as banana and yeast sucrase, turnip peroxidase, and animal tissue lipase and protease, indicate a common property involving enzyme formation or production in the absence of the

[43] Cf. for example, H. v. Euler, "Chemie der Enzyme. I Teil. Allegmeine Chemie der Enzyme," 1920, pp. 291-6.

[44] G. McGuire and K. G. Falk, *Jour. Amer. Chem. Soc.* *45*, 1539 (1923).

[45] R. Willstätter and F. Racke, *Lieb. Ann.* *427*, 111 (1922).

[46] R. Willstätter and A. Pollinger, *Lieb. Ann.* *430*, 269 (1923).

[47] H. M. Noyes, K. Sugiura, and K. G. Falk, *Jour. Amer. Chem. Soc.* *46*, 1885 (1924).

life process and only from materials obtained directly from the living matter. They are of interest as showing possibilities of increases in enzyme activities, at present uncontrolled it is true, which have been assumed, perhaps tacitly, to occur only during life processes. Decreases in enzyme actions are too common to consider in this connection, but such increases as are described compel attention.

The possible changes which manifest themselves in the spontaneous activations of the enzyme preparations may be discussed briefly. They may be considered to consist either in the decomposition or change of some substances present in the materials by which the active enzyme groupings or active enzyme molecules are formed, or the breaking up of compounds of enzymes and inactivating materials which mask the enzyme activities by which the former are removed and the active enzymes produced. Either process would be a chemical transformation accompanied by the appearance of additional enzyme. The bearing of these relations upon the mechanism of the chemical transformations which occur in life processes is of interest.[48]

An important feature of enzyme actions is the specificity, each enzyme catalyzing a more or less definite reaction. Thus, the soy bean urease increases the velocity of the hydrolysis of urea to a very marked extent, but has very little influence on the hydrolysis of methylurea. Sucrase hydrolyzes sucrose and not maltose; maltase the reverse. These examples might be multiplied indefinitely, but for such detailed information the reader is referred to the larger text books dealing with the detailed reactions.

E. Fischer's lock-and-key simile[49] for the mutual getting together of substrate and enzyme, each fitting in with the other, gives a mechanical picture of the action. The actions would evidently depend then upon a combination of enzyme and substrate followed by a breaking down in a different way to regenerate the enzyme and the products of the hydrolysis.

The specificities of enzyme actions have always aroused the greatest interest. The relations have been considered frequently to be peculiarly characteristic of enzyme actions, but the time ap-

[48] Cf. K. G. Falk, "Catalytic Action," Chapter VII, "A Chemical Interpretation of Life Processes."

[49] E. Fischer, *Ber.* 27, 2985 (1894).

pears to be ripe to take a somewhat more moderate view of the question. The specific actions of enzymes vary to a certain extent. Some enzymes, such as urease, are apparently limited in their actions to one substance, others exert their influences on groups of substances. For example, in general terms, α-glucosides are hydrolyzed by maltase, β-glucosides by emulsin, many proteins by pepsin, many peptides by erepsin, many esters by lipase, etc. There are variations with regard to the extent of the actions within each group as well. On the other hand, the specific actions of enzymes, while interesting in every case and striking in many, are not unique among chemical reactions. Systematic qualitative analysis shows many just as interesting and (to the writer) just as striking reactions involving specificity as the reactions of enzyme chemistry. This question will be taken up again in Chapter X.

In reviewing the general relations which are described in this and the preceding chapters bearing upon the physical and chemical properties more or less common to enzyme preparations, it may be asked whether any light has been thrown on the chemical nature of enzymes in general. It may be stated that the general problem appears to revolve about two factors, one the active enzyme, the other the conditions which stabilize the active enzyme so as to maintain its action under different conditions. The active enzyme, as a result of the consideration of the various phenomena, appears to be of the nature of a molecule or part of a molecule which loses its specific property of catalyzing chemical reactions upon very simple treatments. The conditions under which enzymes act, the effects of various reagents upon them especially as they retard the actions, a comparison with the properties of indicators, all suggest that in the inactivations changes occur such as are included in tautomeric transformations. The physical properties of practically all enzyme preparations, even when highly purified, indicate that a complexity of the molecule as shown by the colloidal nature of the material, appears to be essential to the maintenance of the action of the enzyme. The decreasing stability shown by an enzyme upon the progressive removal of inactive substances and the accompanying concentration of active enzyme in a smaller mass, point in the same direction.

If any conclusions as to the nature of an enzyme is permissible upon the basis of the general evidence so far presented, it would be

that an enzyme action is due to a chemical grouping of marked instability present in a complex molecule of colloidal nature. The composition and structure of the molecule would exert an influence in determining the nature of the specificity of the reaction catalyzed, while the complexity of the molecule stabilizes the active enzyme grouping in retarding its rearrangement or decomposition into a more stable and enzymically inactive form.

VI.—Chemical Nature of Certain Enzymes

After treating of the physical and chemical properties which are to a certain extent common to enzyme preparations, the next question in the natural sequence of development would involve the actual chemical nature of certain specific enzymes. It may be stated at once, in order to forestall any misunderstanding, that there is no proof that any enzyme has been obtained in a state of purity as a chemical individual is so considered. The question may even be raised as to whether a chemical molecule possessing a definite molecular weight and arrangement of atoms is present in any enzyme preparation, conferring upon the preparation the properties which are included under enzyme actions. The significance of this statement will be developed further in this chapter.

The first question to be taken up involves the attempts which have been made to obtain enzymes as chemical individuals. Enzyme actions are obtained with materials from biological sources. These materials are almost always colloidal in character. As pointed out in Chapter IV, the purification of such materials or the separation of various constituents without changing their essential properties is extremely difficult, if not impracticable, by the ordinary methods of preparative chemistry. Recourse must be had, therefore, to adsorption procedures, and the results obtained by Willstätter in this way marked a definite advance in such studies.

The increase in purity of an enzyme material is determined as a rule by following the increase in enzyme activity under standard conditions. Such conditions are generally taken to mean conditions for optimum actions. For an enzyme such as peroxidase or yeast sucrase these conditions can be fixed satisfactorily, as foreign substances in moderate concentrations exert little or no influence on the determinations of the action under optimum conditions. On the other hand, for an enzyme such as lipase whose activity is influenced very strongly not only by change in hydrogen ion concentration but also by a number of additional factors, it is difficult to obtain satis-

factory quantitative comparisons to cover different cases.[1] Moreover, the addition of various substances is necessary with some enzymes to obtain comparable reproducible results even at the optimum hydrogen ion concentration. Thus sodium chloride is necessary for the action of pancreatic amylase and calcium chloride for the action of trypsin.

The methods developed by Willstätter, which were outlined in Chapter IV, have given the purest enzyme preparation so far described. In considering the chemical compositions of the products obtained, Willstätter[2] emphasized the fact that when analytical figures are given, they may be of use for purposes of comparison and will give indications of the progress of the preparative study. Analyses are given with the reservation that they are not final results or definite constants, but are meant only to indicate the state of purity attained. Too great significance must not be attached to the analyses, since frequently differences in composition of different preparations of one enzyme are larger than the differences in composition of preparations of different enzymes.

In view of these facts, the elementary compositions of the various enzymes will not be presented in detail. The question of the various constituent groups which may be present is, however, of interest. The positive results which have been obtained in this field are disappointing up to the present in view of the advances which were made in obtaining highly active enzyme preparations.

Some results may be given in order to indicate the present status of the subject. A peroxidase preparation,[3] 12,000 times as active as the original dried turnip from which it was obtained, gave no protein, carbohydrate, or pyrrol reactions, did not form a precipitate with either mercuric chloride or uranyl acetate, gave a faint turbidity with phosphotungstic acid, and formed precipitates with cannin and with iodine-potassium iodide solutions. No reaction which could be referred to a known chemical grouping could be obtained. The iron content was small and did not change propor-

[1] For example, R. Willstätter and F. Memmen, *Z. physiol. Chem. 129*, 1 (1923), added albumin to pancreatic lipase to maintain a constant acidity, or activated the enzyme, with tributyrin as substrate, by the addition of albumin and calcium chloride. The same workers, in another investigation (*Z. physiol. Chem. 133*, 229 (1924)), added sodium oleate, calcium chloride, and an ammonia-ammonium chloride mixture.

[2] R. Willstätter and A. Pollinger, *Lieb. Ann. 430*, 269 (1923).

[3] R. Willstätter and A. Pollinger, *Lieb. Ann. 430*, 269 (1923).

tionately to the activity. Phosphorus was present only as an impurity. The content of nitrogen was in the neighborhood of 10%. With a highly purified pancreatic amylase preparation,[4] no reactions for protein or its decomposition products were found. Millon and ninhydrin tests were also negative, while no turbidity was shown with picric acid or with ferrocyanic acid. A slight test (Molisch) for carbohydrates was observed. In this connection the very careful work of Sherman and his associates, to which reference has already been made, must be mentioned. They found definitely that the various amylases approached more nearly the properties and compositions of proteins, the greater their state of purity. Sucrase preparations were described free from protein, carbohydrate, phosphorus, and all chemically identifiable groups, 1500 times as active as the same weight of yeast initially.[5] The manner of treatment and of purification were shown to have an important bearing upon the character of the impurities present in the sucrase preparations.[6]

These results which were obtained by Willstätter and his coworkers are disappointing in that they do not lead to positive conclusions as to the probable natures of the active enzyme groupings or molecules. It is possible that when larger quantities of the purified preparations are at hand, more definite conclusions will be found.

Some of the results of Euler [7] and his associates on yeast sucrase, purified in part by the methods developed by Willstätter may be presented. Extremely active preparations, which did not increase in activity on further treatments, were studied. It was found that the purest preparations were closely related to the proteins, the state of combination of the nitrogen suggesting in high degree that of the ordinary proteins. Positive biuret, xanthoproteic, and ninhydrin reactions were obtained. The Millon reaction was not typical, a yellow-brown color being produced. The sulfur content, as well as the nitrogen content, were of the order of magnitude of certain protein bodies. Amino nitrogen content was small, but on hydrolysis with hydrochloric acid, two-thirds of the total nitrogen

[4] R. Willstätter, E. Waldschmidt-Leitz, and A. R. F. Hesse, *Z. physiol. Chem.* **126**, 143 (1923).

[5] R. Willstätter and R. Kuhn, *Z. physiol. Chem.* **125**, 28 (1923).

[6] R. Willstätter and F. Racke, *Lieb. Ann.* **427**, 111 (1922); R. Willstätter and K. Schneider, *Z. physiol. Chem.* **133**, 193 (1924).

[7] H. v. Euler and K. Josephson, *Ber.* **56B**, 1097 (1923); **57B**, 299 (1924); *Z. physiol. Chem.* **133**, 279 (1924).

present was given off in this form. These results were followed by the identification and estimation of a number of the amino acids. For preparations showing high activities the following results were obtained:[8] cystine 2%, histidine (or other imidazol bodies) between 2% and 6%, tryptophane 5.5%, tyrosine absent. Crude preparations gave a positive Molisch test which became weaker as the products were purified. However, differences in the typical protein reactions were shown by the sucrase preparations in comparison with those of proteins studied heretofore, so that in speaking of sucrase as a protein, it was meant that protein bodies approached most closely to the properties of highly active sucrase. In this description of sucrase as a "protein-like" substance, Euler and Josephson considered that no contradiction existed with the view of Willstätter and Kuhn who stated that it was possible to obtain sucrase "free of protein, carbohydrate, and phosphorus." The study of the nitrogen component is considered by Euler and Josephson to be of the greatest importance, since, according to their view, enzyme action is associated with the amphoteric properties of the protein (enzyme). Diffusion experiments led to the view that the molecular weight of the sucrase was about 20,000.[9] The ash content of the purest preparations were found to be irregular and bore no relation to the state of purity. The acidic and basic dissociation constants of a purified preparation after 11 days dialysis (0.13% ash) were found to be $k_a = 10^{-7}$, $k_b = 4.3\text{-}5.5 \times 10^{-11}$; calculated isoelectric point (simple formula, Chapter IV) pH 5.0.

Since the study of definite enzymes has not progressed in a manner to permit of their isolation as chemical individuals and identification as such, it will be necessary to proceed along somewhat different lines in order to attempt to obtain some insight into the chemical nature of the compounds upon which enzyme actions depend. The actions of enzymes are markedly dependent upon the hydrogen ion concentration of the media in which they act. It was therefore considered that the active constituents of the enzymes were dependent upon the ionization of the enzyme molecules, that the function of the hydrogen ion concentration in favoring the actions under certain conditions was connected with producing

[8] H. v. Euler and K. Josephson, *Ber. 57B*, 859 (1924).

[9] H. v. Euler, K. Josephson, and K. Myrbäck, *Z. physiol. Chem. 130*, 87 (1923); cf. also H. v. Euler and K. Josephson, *Z. physiol. Chem. 133*, 279 (1924).

favorable concentrations of the enzymically active ion or unionized molecule. Experiments showing the direction of migration in an electric field [10] under conditions of hydrogen ion concentrations at which the enzymes were active gave a number of results indicating that in certain cases one of the ions, in other cases, the unionized molecule, carried the activity. It was found, however, that many of these results were due primarily to the inactive foreign bodies present with the enzymes, that purification of the enzyme materials resulted frequently in a change in the direction of migration,[11] and that, although the experimental evidence showed that in the given cases the enzyme activity was present in the ion or molecule, it did not, in any way, prove that the enzyme activity was dependent upon the existence as such of the ion or unionized molecule. The results on the adsorption of enzymes and their purification in this way are based fundamentally upon the same properties as determined in cataphoresis experiments; aluminium hydroxide adsorbing acid bodies or negative (complex) ions, kaolin adsorbing basic bodies or positive (complex) ions. As indicated in Chapter IV, purification of some of the enzyme materials resulted in changing the characters of the adsorptions. Both adsorption and cataphoresis experiments are based upon the same property, but, in general, neither is directly dependent upon the chemical or physical characteristics of the active enzyme.

In some recent experiments the problem has been attacked from a somewhat different angle with results which promise to throw light upon these difficult questions.[12] The distribution of trypsin inside and outside of gelatin particles at different hydrogen ion concentrations was compared to the distribution of hydrogen and chloride ions under the same conditions. The ionic nature of trypsin was proved in this way, since it was distributed in the concentrations required by the membrane equilibrium equations developed by Willard Gibbs and by Donnan, according to which for a diffusible substance, the ratio of the concentration of any ion on

[10] L. Michaelis, *Biochem. Z. 60,* 91 (1914).

[11] For example, a preparation of purified pepsin showed no direction of migration in an electric field until a protein (albumin or albumose) was added, when it migrated with the protein, assuming its electrical properties (C. A. Pekelharing and W. E. Ringer), *Z. physiol. Chem.* 75, 282 (1911); crude yeast sucrase extract goes to the anode, while the purified sucrase, even at pH 6, goes to the cathode (R. Willstätter).

[12] J. H. Northrop, *J. Gen. Physiol. 6,* 337 (1923-24).

the two sides of a membrane must be equal to the ratio of the concentrations of any other ion of the same sign and valence, whereas a non-ionic substance would be equally distributed on both sides. The ratio of the trypsin concentration in the gelatin to the concentration in the outside liquid was found to be equal to the ratio of the hydrogen ion under the same conditions and to the reciprocal of the chloride ion ratio. These results were found between pH 2.0 and 10.2; at pH 10.2 the trypsin was equally distributed; while on the alkaline side of pH 10.2 the ratio was directly equal to the chloride ratio. It was therefore concluded that trypsin is a positive univalent ion in solutions more acid than pH 10, and a univalent negative ion in more alkaline solutions, and probably isoelectric at about pH 10.2. Unpublished experiments by Northrop lead to analogous conclusions with pepsin.

These results connect more directly the enzyme ions with their substrates and indicate perhaps simpler behaviors involving possibilities of diffusion and combination. In this way chemical relations may be suggested which should prove of great value in the study of the chemical relationship between enzyme and substrate.

In discussing the possible bearing of the ionization of the enzyme substance on its catalytic action and on the nature of the active chemical constituent, the ionization of the substrate must be mentioned in so far as it may influence the reactions. The study [13] of the action of pepsin and of trypsin on a number of proteins brought out the fact that the action was primarily on the protein ion, that the acid salts of the proteins were rapidly attacked by pepsin, the alkali salts by trypsin; that the rates of digestion could be predicted from the amount of protein ion present; that the hydrogen ion concentration for minimum digestion shifted with the isoelectric point of the protein; and that the rates of digestion plotted as functions of the pH were nearly identical with the titration curves of the proteins.

Following the description of some of the conclusions obtained in the study of the ionic nature of enzymes, a few results which have been published in which enzymes were studied more as molecular entities may be quoted.

Some recent work on yeast sucrase published by Euler and his associates indicates interesting possibilities with reference to the

[13] J. H. Northrop, *J. Gen. Physiol.* **3**, 211 (1920-21); **5**, 263 (1922-23).

character of the active enzyme grouping. Inactivation by small amounts of certain metallic salts was studied with the object of determining the smallest amount required to inactivate. Certain groups of organic substances were studied similarly. In this way it was hoped to obtain definite indications of the nature and number of enzyme groupings present which could be inactivated or poisoned. Although conclusive evidence is not at hand as yet, the following results may be quoted. With a partially purified sucrase preparation,[14] the poisoning actions of mercuric and silver salts were reversible quantitatively. Sucrose protected the sucrase against the actions of these salts. Cupric salts were found to be much less poisonous than mercuric and silver salts. With the given preparation and the metallic salts, a gradual regeneration following the initial inactivation occurred. Silver was combined in the sucrase molecule apparently in a manner similar, as far as could be told by electrometric measurements, to its combination in egg albumin, cystein, or a nucleic acid. With a very much purer preparation [15] at pH 4.5 with silver nitrate a limiting poisoning action was found, 25% of the sucrase retaining its activity. Similar results were obtained with mercuric salts, although here disturbing influences obscured the relations somewhat. The inactivating actions of a number of amines were found to correspond to their affinity for formaldehyde in the formation of Schiff bases. Paraphenylene diamine exerted an exceptionally marked inactivating action. Hydrocyanic acid, on the other hand, inhibited the action only slightly.[16] The actions of a number of additional substances were studied, similarly, including bromine and iodine.[17] The hydrogen ion concentration exerted a marked influence in a number of these actions, and had to be carefully controlled. Although no definite conclusions were presented, the results are suggestive with reference to the further study of the chemical nature of sucrase.

Reference may be made in this connection to a view which considers that the actions of peroxidase, catalase, reductase, and amylase, are due to an aldehyde group, and that formaldehyde may be

[14] H. v. Euler and O. Svanberg, *Fermentforschung 3*, 330 (1919-20); *4*, 29, 54, 142 (1920-21).

[15] H. v. Euler and K. Myrbäck, *Arch. f. Kemi, Mineral. Geol. 8* (1922), *Z. physiol. Chem. 121*, 177 (1922).

[16] H. v. Euler and K. Myrbäck, *Z. physiol. Chem. 125*, 297 (1923).

[17] H. v. Euler and K. Josephson, *Z. physiol. Chem. 127*, 99 (1923).

taken as the chemical model for these four types of enzymes since it is stated that it shows all these types of activity.[18]

A direct method of study similar to those just quoted was published recently by Marston.[19] Proteases, including trypsin, erepsin, pepsin, and papain, were found to combine with and be precipitated by safranine and in general azine bases (indulines, etc.). The proteolytic powers were recovered under conditions favorable to the dissociation of the azine-enzyme compounds, such as increase in acidity. The acid nature or property of the enzyme, in every case, was responsible for the combination which took place with the basic nitrogen of the azine nucleus. It was also suggested that "instead of the conventional structure, the protein molecule may be considered to be built up of a series of aminoacid anhydrides, or, in other words, protein has essentially a polydiketo-piperazine structure. . . . Such a structural configuration of the protein molecule would make quite clear the absence of terminal NH_2 groups. Peculiarities of protein ionization and their neutralizing power may be easily explained by means of this suggested structure. The similarity of each ring to the azine nucleus of the bases which are functional in combining with the enzyme suggests that the N in these groups is the seat of action of the enzyme."

These results are interesting in that more definite compounds with enzymes appear to have been obtained than heretofore. Further work along these lines is promised.

The whole problem may also be attacked in a somewhat different way. An enzyme, as a rule, catalyzes a more or less specific reaction or group of reactions. Considering the very complex nature of the protein or other molecule which includes the enzyme, or with which the enzyme may be associated, and the more or less specific reaction which it influences, it would appear, as suggested in the last chapter, that a reasonable assumption would consider that some definite grouping in the complex enzyme molecule is responsible for a given enzyme action. The problem would therefore resolve itself from this point of view into a study of the chemical nature of such a grouping.

The enzyme lipase was studied in this way, and the results

[18] G. Wöker and H. Maggi, *Ber. 52*, 1594 (1919); H. Maggi, *Fermentforschung, 2*, 304 (1919). Cf. also E. Rona, *Biochem. Z. 109*, 279 (1920) and P. H. Gallager, *Biochem. J. 18*, 29, 39 (1924).

[19] H. R. Marston, *Biochem. J. 17*, 851 (1923).

found are of interest as showing a possible solution of the chemical nature of this enzyme. Some space will therefore be devoted to this phase of the problem and the attempt at its solution.[20]

This lipase work was carried out in the main with preparations from castor beans, although other sources were also used. There has been a general tendency in the study of enzyme actions to attempt to attain conditions under which the enzyme would show a maximum action. This method of studying the problem is likely to introduce a number of new complicating factors, so that it was considered that if the action was due to some definite grouping, a study of the factors which caused a loss of the action might aid in throwing light on its nature. A systematic study of the factors which caused inactivation of the esterase and lipase was therefore undertaken. The results were presented in detail elsewhere.[21]

Inactivation of lipase and esterase preparations was brought about by acids, bases, neutral salts, alcohols, acetone, esters and heat.

The different ways in which these preparations may be inactivated make it appear at first sight as if different reactions occur in the inactivations. If, however, a definite chemical group is responsible for a definite enzyme action, it might perhaps be more reasonable to assume that inactivation follows a definite reaction. The preparations used were essentially protein in character. There was no evidence that a dehydration, or loss of the elements of water, caused inactivation. Some of the reactions indicated that a possible hydrolysis might be a cause of inactivation. With proteins, hydrolysis is generally taken to occur with the $-CO-NH-$ group, the peptide linking, which goes over into the carboxyl and amino groups. Experiments with all the inactivations in no case showed an increase in the formol titration such as would be expected in this reaction, and therefore makes the assumption of such a hydrolysis improbable. Coagulation of the material accompanied some of the inactivations. This physical change alone does not appear satisfactory as an explanation; some change in chemical structure unquestionably must accompany or produce the physical phenomenon. Also, the lipase material in water suspension showed the same activity as in 1.5 normal sodium chloride solution.

The explanations of the chemical changes accompanying inactiva-

[20] K. G. Falk, "A Chemical Study of Enzyme Actions," *Science* 47, 423 (1918).
[21] *J. Biol. Chem.* 31, 97 (1917).

tion so far suggested are not satisfactory. The reagents used are simple. It is difficult to conceive of a very deep-seated chemical reaction taking place under so many different conditions, none of a complex nature. The only chemical change which appears probable under these conditions is that involving a simple rearrangement within the molecule, such as a tautomeric (or perhaps better, desmotropic) change involving in the simplest case the change in position of a hydrogen atom. In considering the structure of proteins it is evident that such a rearrangement is possible in the peptide linking.

The hypothesis suggested is that the active grouping of the esterase and lipase preparations is of the enol-lactim structure, $-C(OH)=N-$, the specificities being dependent in part upon the groups combined with the carbon and nitrogen, and that inactivation consists primarily in a rearrangement to the keto-lactam group, $-CO-NH-$.

This hypothesis was tested in several different ways. It has been found that in tautomeric substances, the presence of alkali in solution favors the enol form of compounds showing such tautomerism, while acid favors the existence of the keto form. The hydrolytic actions of some simple dipeptides on esters at different hydrogen ion concentrations would, therefore, be evidence bearing on this point, the alkaline solutions presumably favoring the enol-lactim structure. In order to find the actions exerted by the amino-carboxyl groups of the peptide, the hydrolytic actions of a number of aminoacids on different esters were determined under similar conditions at the same hydrogen ion concentrations. The actions of the dipeptides and aminoacids were also measured with the actions of the amino-carboxyl groups masked by the hydrogen of the carboxyl group being replaced by the ethyl group, and also by testing compounds such as hippuric acid, which do not contain an amino group.

In these compounds, it is possible that the equilibrium between the keto-lactam enol-lactim forms might be changed rapidly if the conditions were changed slightly. A more stable substance was therefore studied from this point of view. Imido esters, as shown by the formula (*a*), possess the enol-lactim structure in which the hydrogen atoms may be substituted by organic radicals. The hydrolytic actions on esters of ethyl imidobenzoate (*b*) at different

hydrogen ion concentrations and under various conditions were measured.

$$\underset{\text{(a)}}{R-C(OR')=NR''}, \qquad \underset{\text{(b)}}{C_6H_5-C(OC_2H_5)=NH}$$

Finally, in order to reproduce the conditions and properties of naturally occurring lipases as far as possible, a number of different proteins were treated with alkali for the purpose of producing an enol-lactim grouping in the peptide linking if this were possible, then neutralized to different hydrogen ion concentrations and the hydrolytic actions tested on a number of different esters.

The following table (p. 130) shows some of the experimental results obtained. The determinations were made by titration with alkali, all necessary corrections for blanks being introduced. The actions were then calculated in terms of equivalents of acid $\times 10^4$ (as titrated with 0.1 N alkali solution) formed from 1 gram equivalent of the ester by the action of 0.1 gram substance in 24 hours at 38°. The relative results were then calculated with the ester showing the greatest amount of hydrolysis put at a value of 100.

These results show one of the most striking characteristics of the actions, that is, their selectivity. Different substances showed markedly different actions on the esters. It is not surprising in the first instance to obtain hydrolytic actions, but the variations are interesting. The chemical configurations may also be considered somewhat further. In doing this, the further experimental results found will not be given in detail but will only be referred to.[25]

With regard to hydrolytic actions of a number of the simpler peptides on esters, the actions were very much more marked in the alkaline solutions than in the neutral. At pH 9.0, for example, considerable action was obtained, especially toward the acetates. There was in these cases a decrease in the hydroxyl ion concentration in the course of the experiments, approaching neutrality or going beyond in some cases. It is difficult to judge how far this influenced the results, as it undoubtedly did. The objection may be raised that the alkalinity of the solutions alone caused the hydrolysis, and that the peptides acted only as buffer mixtures to keep the hydroxyl ion concentration predominant as compared to the water-ester blanks, which in some cases became neutral or slightly

[25] For experimental details and data cf. K. G. Falk, *J. Biol. Chem.* *31*, 97 (1917).

Selective Action of Ester-Hydrolyzing Substances

	Glycine [22] pH 6.1	Castor Beans [23]	Glutamic Acid [22]	Aspartic Acid [22]	Glycyl glycine [23] 9.0	Imido Benzoate [23] 7.0	Casein [24] 8.0	Gelatin [24] 8.0	Egg Albumin [24] 9.0
Phenyl acetate	100	100	30	16	100	100	0	46	100
Methyl acetate	44	38	100	100	60	90	37	36	46
Glyceryl triacetate.	71	82	50	59	24	36	49	28	85
Ethyl acetate	83	22	72	79	46	83	36	39	0
Ethyl butyrate	20	48	17	11	15	33	0	4	0
Methyl benzoate ..	..	..	..	..	5	14	100	0	0
Ethyl benzoate	19	6	12	6	0	0	0	100	68
Phenyl benzoate ...	5	3	4	4	0	0	..	..	..
Olive oil	..	0	..	..	..	..	2	11	0
Cotton seed oil.....	..	..	..	..	..	..	0	..	0
Castor oil	..	6	..	..	..	..	..	..	..

[22] M. L. Hamlin, *Jour. Amer. Chem. Soc. 35,* 1897 (1913).
[23] K. G. Falk, *J. Biol. Chem. 31,* 97 (1917).
[24] F. Hulton-Frankel, *J. Biol. Chem. 32,* 395 (1917).

acid in reaction more rapidly. This objection is met by comparison with the results obtained with the aminoacid solutions where the buffer action was essentially the same, but entirely different actions were obtained, both absolutely and relatively. A comparative study of the hydrolytic actions of the dipeptides in themselves also meets the objection.

The general formula for the peptides may be written as follows:

$$\begin{array}{l} CHR-CO-NH-CHR' \\ \quad | \qquad\qquad\quad | \\ NH_2 \qquad\quad CO_2H \end{array} \quad \text{or} \quad \begin{array}{l} CHR-C(OH)=N-CHR' \\ \quad | \qquad\qquad\quad | \\ NH_2 \qquad\quad CO_2H \end{array}$$

The groups which may be considered to be involved in the hydrolytic actions are the amino and carboxyl groups or the central — CO — NH — group or its tautomer. In attempting to separate the actions of these groups, two lines of experimentation were followed. In the first place, the actions of the amino and carboxyl groups were masked by using glycylglycine ester hydrochloride and hydrobromide; and secondly, the actions of the amino and carboxyl groups alone were followed by studying aminoacids, all under the same conditions under which the peptides were studied. While these methods permit the studying of the groups alone, they leave out of account the possible factor of the influence on the tautomeric equilibrium of the molecule as a whole. The results showed very marked actions at pH 8 and 9, but the solutions became neutral rapidly (presumably in this way reverting back to the keto-lactam structure). A significant feature was the lack of action toward ethyl butyrate, and the comparatively large action toward glyceryl triacetate.

The second method of studying the influence of the different groups separately was to compare the actions of some aminoacids with the peptides under similar conditions. This may be illustrated by comparing the following formulas for aminoacids and peptides.

$$\begin{array}{l} \text{(A)} \\ CHR-CO-NH-CHR' \\ \quad | \qquad\qquad\quad | \\ NH_2 \qquad\quad CO_2H \end{array} \qquad \begin{array}{l} \text{(B)} \\ CHR \\ \quad / \quad \backslash \\ NH_2 \quad CO_2H \end{array}$$

By comparing the actions of equivalent amounts of substances of Formulas (A) and (B) under comparable conditions, it should be possible to find the action due to the grouping — CO — NH in (A)

with the possible reservation that this group and the amino and carboxyl groups may exert reciprocal influences upon each other although no direct evidence of such influence has been obtained. The results obtained in an extended series of experiments led to the following conclusions: Under the conditions used the ratios of the actions toward glyceryl triacetate and ethyl butyrate of the simple aminoacids was close to unity, while for the dipeptides it varied from 5 to 12 to one. This proves that the actions were not due to the hydroxyl ion concentrations, but that the aminoacids and peptides are the important factors. The hydrolytic action of one gram molecule of glycine toward the different esters was calculated from the experimental results and may be given in terms of the amount of acid in tenths of millimols formed at 38° from 1.0 c.c. of ethyl butyrate, 0.5 c.c. of glyceryl triacetate, etc. Toward ethyl butyrate for 26 hours' action it was found to be 6.1×10^2; for 45 to 46 hours 8.2×10^2 (mean); toward glyceryl triacetate it was found to be 8.4×10^2 for 26 hours' action, and 10.8×10^2 for 45 to 46 hours. With dipeptides, the mean actions found for 19 hours were 6.2×10^2 toward ethyl butyrate and 46.4×10^2 toward glyceryl triacetate. This indicates that the action of the dipeptides toward ethyl butyrate was due mainly to the amino and carboxyl groups and confirmed the results obtained with the glycylglycine ester hydrogen halides. Subtraction of the aminoacid glyceryl triacetate value from the value of the peptide left an action of 35.8×10^2 to be accounted for by the group $-CO-NH-$ or $-C(OH)=N-$. The mean value found with the peptide ester hydrogen halides was 17.2×10^2 but the difference may well have been due to the more rapid neutralization and accompanying shift in the tautomeric equilibrium with the latter.

The actions of the simpler aminoacids toward different esters were treated in detail by Hamlin [26] and it was shown by him that if the esters were arranged in a series according to the extent of their hydrolyses, different arrangements resulted with the different aminoacids for the same hydrogen ion concentration and different as well from an isohydric solution containing no aminoacid.

Since the keto-lactam group is present in other substances besides peptides, experiments were carried out to find whether these exerted any hydrolytic actions on esters. Urea gave no action

[26] M. L. Hamlin, *Jour. Amer. Chem. Soc.* 35, 1897 (1913).

whatsoever at hydrogen ion concentrations between 10^{-4} and 10^{-10} N. Hippuric acid gave very small actions at $10^{-9.0}$ N. It is therefore evident that the structure of the compound as a whole is of importance in determining the equilibrium between the tautomeric forms, if these be involved in the actions.

In the table, the results with an imido ester were given. Imido esters contain the enol-lactim structure, possessing the general formula R — C(OR′) = NR″. The one studied in this work was ethyl imidobenzoate, $C_6H_5 — C(OC_2H_5) = NH$. The action toward ethyl butyrate was found to be small. The actions toward glyceryl triacetate were comparatively large, however. A maximum action was observable at pH 8, compared with more acid or more alkaline solutions. The results toward different esters calculated as for the dipeptides, at pH 7.0 gave the same order of decreasing action as for glycylglycine at pH 9.0, except that the positions of the first two members, glyceryl triacetate and phenyl acetate, were interchanged. This shows a marked similarity in behavior, while the minor difference may be due to secondary differences in structure. The results toward glyceryl triacetate with the imido ester, especially at pH 7.0 to 10.0, were not far removed from the results for the dipeptides at pH 9.0. Since the action toward ethyl butyrate was small or negligible, it is evident that the actions observed confirm the view that the action is due to the grouping — C(OR) = N —.

An interesting parallelism between the imido ester and the naturally occurring lipases is the fact that the former is inactivated by acids, by bases, by heating in solution, and by long standing in solution. This inactivation refers to the ester-hydrolyzing action and the change is accompanied chemically by the hydrolysis of the (OR) grouping followed by the tautomeric rearrangement of the amidine complex to the acid amide, and probably hydrolysis of the latter. The optimum hydrogen ion concentration for the imido ester is also interesting. This may be due to its more rapid decomposition in more acid or more alkaline solutions.

In order to determine whether or not the conditions which are known to favor the enol-lactim grouping in simple substances will produce ester-hydrolyzing groups or substances from proteins, an investigation was carried out in which a number of proteins (fifteen in all) were treated with alkali of different strengths and under different conditions, and after neutralization to various points tested

for ester-hydrolyzing actions.[27] Some of the results are shown in the table and the general conclusions arrived at were as follows: The time and temperature at which the alkali stood in contact with the protein did not seem to make much difference in the activity of the solution except where the temperature was quite high (80° C.). With regard to the concentration of alkali, for casein, gelatin, and egg albumin, 3 N alkali seemed to produce solutions of highest activity. The solutions showed tendencies toward optimum acidity conditions, though not very marked. Activity was greater in slightly alkaline solution. When hydrolysis of the protein was accomplished by acid instead of by alkali, the solutions, when treated similarly, did not possess ester-hydrolyzing properties.

In this discussion of the active grouping in lipase actions, the experimental work was limited almost entirely to the peptide linking occurring in proteins. It is evident, however, that such tautomeric or desmotropic structures, enol-lactim and keto-lactam, may be present possibly with the hydrogen of the hydroxyl substituted by a hydrocarbon or other radical in other groupings, and the results of this investigation in no way limit the activity to the peptide linking. In view of the complexity of the protein molecule, it is highly probable that such structures are present and rearrangements possible with other groups and that the specificities are in part dependent upon these.

It may be pointed out in this connection that in a paper entitled "The Catalytic Action of Amino-Acids, Peptones, and Proteins in Effecting Certain Syntheses,"[28] Dakin showed that the velocities of a number of condensation reactions of organic substances, some of them possibly analogous to some occurring in the living cell, are increased by aminoacids, peptones, albumoses, and even proteins as catalysts. This work is most suggestive and makes it appear probable that many more such reactions will be found, especially now that conditions can be controlled more satisfactorily than when Dakin carried out his experiments.

In how far the conclusions reached with lipase may be applied to other enzymes is a question. It seems probable, because of the comparatively simple treatments by which most enzymes may be inactivated and also activated, that with them also a simple arrange-

[27] F. Hulton-Frankel, *J. Biol. Chem.* **32**, 395 (1917).
[28] H. D. Dakin, *J. Biol. Chem.* **7**, 49 (1909-1910).

ment or perhaps desmotropic change is connected with loss or gain in activity. There is, however, no reason to suppose that the active grouping is the same for all enzymes. Each enzyme must be studied separately and the conclusions as to the chemical nature of one active enzyme grouping cannot, without further evidence, be applied to an enzyme grouping connected with a different action.

The fact that the hypothesis with regard to the active grouping in lipolytic action gave positive results must be looked upon as a fortunate guess. Though there is no direct proof that the active lipase grouping is the one indicated, a number of indirect lines of evidence point to the same conclusion and this group may be accepted as the enzymatically active one, until there is a more satisfactory explanation, or direct evidence to the contrary. The analogy of the imido ester and the naturally occurring lipases is striking. Unquestionably a study of the hydrolytic actions of a number of different imido esters and their substitution products upon different esters, will give results showing as great differences and specificities as the naturally occurring lipases.

Further, it seems fairly certain from the evidence presented here and in Chapter V, that inactivation of lipase preparations, and in all probability of all other enzyme preparations, depends upon simple tautomeric changes or rearrangements, reversible in some cases, irreversible in others, within the molecule, and that the specificity depends in part upon the various atoms or groups, their natures and arrangements, combined with the active grouping, and to a minor extent upon those atoms or groups present in the rest of the molecule, and in part possibly upon the action of the substrate upon the enzyme preparation as will be developed in the following chapter.

The view that an enzyme preparation is made up of or contains an active grouping or substance, unstable to a certain extent, which is stabilized by another grouping or substance, possibly colloidal in nature, was suggested earlier by Perrin [29] and also by Röhmann and Shmanine [30] in connection with peroxidases. Mathews and Glenn [31] considered enzymes to be a combination of a colloid with an active principle. The former was thought to be related in nature to the substrate, while the latter in the case of sucrase and diastase was

[29] J. Perrin, *J. Chim. Phys.* *3*, 50 (1905).
[30] F. Röhmann and T. Shmanine, *Biochem. Z.* *42*, 235 (1912).
[31] A. P. Mathews and T. H. Glenn, *J. Biol. Chem.* *9*, 29 (1911).

considered to be protein. A similar point of view has been emphasized repeatedly by Willstätter as a result of his recent work on enzymes, as, for example, when he states[32] that "a molecule of an enzyme consists of a colloidal carrier and a purely chemically active group."

Some facts which should perhaps be taken up in the following chapter in connection with the "Mechanism of Enzyme Actions" will be given now as they may aid in throwing light on the chemical nature of enzymes. In any event, they form a transition discussion for the material next to be presented.

An extended investigation on the amylases from typical animal, vegetable, and fungus sources has been carried out by H. C. Sherman and his co-workers.[33] Fractional precipitation with alcohols of different strengths and with ammonium sulfate and dialysis were the methods employed in the purification. While the products obtained were very active, they were not as active as the best of the preparations obtained by the use of the adsorption procedures developed by Willstätter. The methods of determining the actions of the amylases on soluble starch were studied carefully by Sherman. The amyloclastic (starch splitting, disappearance of blue color with iodine) and saccharogenic (formation of substances reducing cupric salts) actions were compared.[34] Differences found by the two methods of testing were doubtless due to the fact that "saccharogenic power" refers to the amount of maltose produced and "amyloclastic power" refers to the amount of starch all of which is digested to a certain point within a certain time. The latter method may therefore giving misleadingly low results. A number of starches from different sources were also studied but showed no differences in behavior.[35]

With regard to the two methods of testing, the malt enzyme showed that the iodine reaction persisted even after much maltose had been formed. For instance, after one-half of the original weight of the starch had been transformed into maltose, addition of iodine gave a deep blue color, after two-thirds, the iodine test was violet

[32] R. Willstätter, *Ber. 55B*, 3601 (1922).

[33] Series of papers published in *Jour. Amer. Chem. Soc.* and summarized in *Proc. Nat. Acad. Sci. 9*, 81 (1923).

[34] H. C. Sherman and M. D. Schlesinger, *Jour. Amer. Chem. Soc. 35*, 1784 (1913).

[35] H. C. Sherman, F. Walker, and M. L. Caldwell, *Jour. Amer. Chem. Soc. 41*, 1123 (1919).

blue to violet red. On the other hand, with the pancreatic enzyme the iodine test was red when two-fifths to one-half of the original weight of the starch had been transformed into maltose, and no color shown after one-half to three-fifths. The *Aspergillus oryzae* amylase, on the other hand, possessed greater amyloclastic and smaller saccharogenic actions than did the active malt preparations.

It may be stated that the actions of salt on the activities of these amylase preparations were also studied carefully. For example, the removal of salts did not inactivate the malt enzyme completely as it did the pancreatic enzyme.[36] Further, with malt, the amyloclastic action reaches an optimum at concentrations of the activating agent (salt) much below that which gives the optimum saccharogenic action. In other words, the former action requires a smaller salt concentration for a maximum than does the latter action.

The separation of the esterase and lipase materials in castor beans may be referred to briefly.[37] The former is soluble in water, insoluble in moderately concentrated salt solution, the latter insoluble in water, soluble in salt solution. To obtain the desired enzymes, husk and oil free castor beans may be extracted with water, dialyzed and filtered. A clear solution is obtained in this way. The longer the time of dialysis and the higher the temperature of the water, the greater the loss in activity. Precipitated with acetone, the solid preparation showed a certain activity in some cases when dissolved again. The residue from the water extraction was extracted with 1.5 N sodium chloride solution. More concentrated salt solution gave a larger amount of extracted material but smaller enzyme action. The salt extract was dialyzed. Length of time of dialysis had no effect on the activity. The precipitated material contained the active enzyme, but if this was filtered, washed with acetone or alcohol, all activity was lost. Sodium fluoride retarded the actions, other salts, including manganous sulfate, had no effect. Both preparations were essentially protein in character.

The actions of these two preparations under comparable conditions on ethyl butyrate and glyceryl triacetate gave results which indicated that the water soluble enzyme may be designated as an esterase, the salt soluble as lipase. Both preparations acted on both esters, the difference being one of relative amount of action.

[36] H. C. Sherman and A. W. Thomas, *Jour. Amer. Chem. Soc.* 37, 623 (1915).
[37] K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc.* 37, 217 (1915).

It may also be mentioned that the water insolubility of the castor bean lipase does not mean that all lipases show this property. For instance, the soy bean lipase was found to be soluble in water.[38] On the basis of the views already developed, this would mean only that the active enzyme grouping is combined or contained in different complexes which show varying properties in the two cases.

The description of the various enzyme preparations or modes of action in the latter part of this chapter does not add to the knowledge of the chemical nature of the active enzymes. The methods used in the different cases follow closely those used in obtaining substances of biochemical origin in general. It is possible with enzyme preparations to keep a close check on the changes which may take place in the various manipulations by means of the enzyme actions, which, as already pointed out, may reflect changes not detected by the more usual chemical methods. On the other hand, the choice of reagents which may be used is also limited to a certain extent by their possible action on the enzyme.

A practical point in such methods must not be overlooked. The possibility of bacterial growth in the preparations must be excluded rigidly, both in obtaining enzyme material and in testing its activity as otherwise it would be impossible to determine whether a given chemical reaction is due to bacteria or to the enzyme under investigation. Since it is extremely difficult to keep the material which is generally used in a sterile condition, it is necessary to employ substances which will prevent bacterial growth. A great number have been suggested and used including toluene, chloroform, tricresol, various salts such as sodium fluoride, cyanides, etc. In every case, the possibility exists that the substance added as bactericide or antiseptic may exert a deleterious action on the enzyme, possibly inactivating it. The special substance to be used in any one enzyme investigation must be carefully scrutinized, therefore, at the same time that the use of some such agent must be looked upon as imperative.[39]

[38] K. G. Falk, *Jour. Amer. Chem. Soc.* **37**, 649 (1915).
[39] Cf. S. Morgulis and V. E. Levine, *Science* **52**, 202 (1920).

VII.—Mechanism of Enzyme Actions

Enzyme actions manifest themselves in the changes in velocity of definite chemical reactions brought about by the presence of certain preparations of biochemical origin. They may be considered to be a group of catalytic reactions. The development of the subject of chemical kinetics has shown the methods by which the velocity of a chemical reaction may be measured and used in connection with mathematical expressions, and general mathematical equations deduced which show the law covering the course of the velocity of the change. In Chapter II some of the simpler kinetic equations were given with illustrations of their uses, and an attempt was made to show some of the fundamental concepts upon which the deductions and applications of these equations depend, as well as the possible complications which may arise in their use.

The mechanism of enzyme actions may include such relations as the various steps if the reaction takes place in stages, the factors influencing the velocity of the reaction, the type of product formed, etc. The actual chemical composition and chemical properties of any of the substances taking part are not involved primarily in the discussion. They are, of course, directly responsible for any and all of the phenomena observed in reactions. The main factors to be considered here, however, are the velocities of the chemical reactions, the factors which influence these, and the light which these relations may throw upon the enzyme actions involved.

The first step in this discussion would be an attempt to apply the equations of reaction velocity given in Chapter II to enzyme actions, since enzymes manifest themselves by causing changes in such velocities. As shown in Chapter III, the further careful study of reaction velocities has resulted in finding that a number of apparently simple chemical reactions, whose changes agreed with the simple kinetic relationships, when studied more carefully showed variations which necessitated further theoretical treatment and explanation. Although the simple kinetic laws cannot therefore be

applied and be expected to hold in as satisfactory a form as was thought for a considerable period of time, they should serve as a first attempt to examine the velocities of reactions as influenced by enzymes.

One of the most important uses of the reaction velocity determinations in enzyme actions is in connection with comparing the activities of preparations from different sources and in different states of purity. It is necessary to have such units of measurement based upon reaction velocities since enzymes, as yet, are not at hand as pure substances. The most obvious method of comparing the activities of a definite enzyme is by means of the reaction velocity constants. While this is possible in some cases, as a rule such simple comparisons are impracticable, due in part to the frequent change in value of the constant as the reaction proceeds, to the marked influence of various substances including foreign bodies as well as products of the reaction on many of the actions, to the gradual spontaneous loss of activity of a number of the enzyme preparations, and to other causes. Attempts have been made by various workers to set up standards of comparison of enzyme actions, which, although at times empirical, are frequently extremely useful.

Recently, Willstätter and Kuhn [1] suggested certain arbitrary units of measurement or standards for comparison for the enzymes studied by them. Two sets of units were suggested: "enzyme units" which indicate the amounts of enzyme giving a definite chemical change, and "enzyme values" which give the number of enzyme units in definite amounts of substance. For example, the lipase unit was chosen to be the quantity of enzyme material which would hydrolyze 24% of a 2.5% olive oil emulsion in 1 hour under certain standard conditions, and the lipase value would then be the number of lipase units in 1 cg. of material; the amylase unit was taken as 100 times the amount of enzyme material for which the reaction velocity constant $k = 0.01$, and the amylase value as equal to the number of amylase units in 1 cg. of substance; the peroxidase unit as the amount of enzyme material which gives a reaction, equivalent to 1 gram of purpurogallin number 1, etc. These units are extremely useful especially in following the increase in purity of various enzyme preparations and the effects of added substances, although

[1] R. Willstätter and R. Kuhn, *Ber.* *56B*, 509 (1923).

it must be remembered, especially for an enzyme such as lipase, that the choice of substrate and conditions of action are arbitrarily fixed.

The kinetics of the sucrose hydrolysis, especially by yeast sucrase, has been studied more carefully than any other enzyme action. The results obtained bring out a number of relations which should be of general applicability, and will therefore be given in some detail. In the hydrolysis of sucrose, the optical rotation changes from dextro to levo in the course of the reaction. As indicated in Chapter III, the reaction may be quite complex when the possibility of the isomeric α and β and perhaps other forms of the hexoses are involved. In the enzymic decomposition of sucrose, the α forms only are formed directly. They gradually go over into an equilibrium mixture of α and β forms (mutarotation). In the experimental determination of the enzyme action, after suitable time periods, the actions are stopped and equilibrium mixtures of the sugars formed by the addition of sodium carbonate solution.

In order to compare the activities of different sucrase preparations, the method suggested originally by O'Sullivan and Tompson[2] is still generally employed. The time in minutes which is required at 20° with 0.05 gm. of the preparation dissolved in 5 cc. of 0.5 N monosodium phosphate solution and added at 20 cc. of a 20% sucrose solution, for the rotation to attain 0° for sodium light is taken as the measure of the enzyme. Obviously, the more active the enzyme, the smaller the time value. For the given conditions, to reach the rotation of 0° necessitates hydrolysis of 75.93% of the sucrose.[3] The purest or most active sucrase preparations so far described gave time values of very nearly 0.2 minute.[4] Willstätter and Racke, in the experimental determinations of time values used sucrase concentrations such that the time required for 0 rotation ranged from 60-180 minutes. Willstätter and Kuhn defined the sucrase unit as the amount of enzyme in 50 mg. of material having the time value of 1 under the indicated conditions. The sucrase value is given by them as the reciprocal of the time value, that is, as the number of sucrase units in 50 mg. substance. In the experimental determination of the enzyme unit, the fact that the reaction velocity constants are found to vary somewhat, as will be shown

[2] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc. 57,* 834 (1890).
[3] R. Willstätter and K. Schneider, *Z. physiol. Chem. 134,* 193 (1924).
[4] H. v. Euler and K. Josephson, *Ber. 57B,* 299 (1924).

presently, is of secondary importance in so far as the absolute values of the numbers representing the activities are concerned.

Euler [5] has suggested and used a slightly different system of units for the action of the sucrase.[6] The inversion constant, k, for a sucrose solution of definite concentration is obtained from the equation

$$k = \frac{1}{t}\log_{10}\frac{R+L}{\alpha+L}$$

in which R denotes the initial rotation, L the final rotation, and α the rotation at time t (minutes). The activity number is found by dividing the inversion constant by the percentage concentration of the enzyme preparation. The inversion ability, *If*, a unit used extensively by Euler,[7] is given by the equation

$$If = \frac{k \times g\ (\text{sucrose})}{g\ (\text{preparation})}.$$

The relation of O'Sullivan and Tompson's time value unit $0° = t$ minutes (for 4 gm. sucrose and 0.05 gm. enzyme preparation) to Euler's unit is given by the equation [8]

$$If = \frac{46.176}{t}.$$

For the purest preparations obtained by the adsorption procedures, the *If* values may be said to have ranged from 230 to 250,[9] or perhaps even higher in some cases.[10]

These or similar units are necessary for the more careful comparative studies of enzyme actions. That they may be based upon relations or deductions which are not necessarily exact and that the values obtained for constants fluctuate at times are matters of secondary importance in the comparative actions. These

[5] H. v. Euler, "Chemie der Enzyme, II Teil. Specielle Chemie der Enzyme," 1922, pp. 201-2.

[6] An analogous unit was suggested for amylase, H. v. Euler and O. Svanberg, *Z. physiol. Chem. 112*, 193 (1920); *115*, 179 (1921).

[7] H. v. Euler and O. Svanberg, *Z. physiol. Chem. 106*, 201 (1919).

[8] This equation refers to 15.5°. Since the temperature coefficient of the inversion velocity is approximately 10% per degree, at 18° the equation would be

$$If_{18°} = \frac{58.6}{t}, \text{ and at } 20°, If_{20°} = \frac{69.1}{t}.$$

Cf. H. v. Euler and K. Josephson, *Ber. 56B*, 1749 (1923).

[9] K. Myrbäck, *Arkiv f. Kemi, Minerol. Geol. 9, No. 2* (1924).

[10] R. Willstätter and K. Schneider, *Z. physiol. Chem. 133*, 193 (1924).

fluctuations and variations may, however, be extremely significant in the study of the possible mechanism of enzyme actions. Some of the results and conclusions obtained with yeast sucrase will therefore be presented. The hydrolysis of sucrose by acids was considered in Chapter II.

The velocity of the reaction with sucrase has been measured repeatedly by a number of different workers. Contradictory results were obtained at times, but in one of the more recent investigations [11] in which conditions were rigidly controlled, the results were put in a more satisfactory light and certain definite conclusions with regard to the kinetics of the reaction were arrived at.

In the first place, Nelson and Vosburgh applied the monomolecular velocity equations to the reaction at 37° and at different degrees of acidity (hydrogen ion concentrations). With optimum conditions of acidity, pH 4.5, it was found that the value of the velocity coefficient, k, increased as the reaction proceeded.[12] With lower hydrogen ion concentrations, the value of the velocity coefficient was very nearly constant. Results were given for 24 experiments. Of these, 19 gave increasing values for the velocity coefficients, 5 gave practically constant values. This shows that the reaction did not take place according to the monomolecular law, but appeared to do so under certain conditions. Where decreasing values have been found, destruction or inactivation of the sucrase during the progress of the reaction undoubtedly occurred.

With regard to the relations between the concentrations of sucrase and sucrose and the velocity of reaction, it was found that, using as a measure of comparison the time required to cause a certain change (say 40%), the velocity of hydrolysis was directly proportional to the concentration of the sucrase, but not proportional to the sucrose concentration. In fact, within certain limits it was found to be independent of the sucrose concentration. With a certain sucrase solution, the velocities for the change up to 25% inversion with sucrose solutions of different concentrations, showed (for the relation between original concentration of sucrose and time for 25% inversion) increasing velocities with increase in sucrose concentration up to a concentration of 5%, and above that constant velocity.

[11] J. M. Nelson and W. C. Vosburgh, *Jour. Amer. Chem. Soc. 39*, 790 (1917); W. C. Vosburgh, *Dissertation, Columbia University, 1919.*

[12] Cf. R. Willstätter, J. Graser and R. Kuhn, *Z. physiol. Chem. 123*, 1 (1922) for similar results.

This was found repeatedly and may evidently be interpreted in the sense that sucrase possesses a definite capacity for increasing the velocity of the sucrose hydrolysis reaction. That is, sucrase can increase the velocity of hydrolysis of a definite amount of sucrose in a unit of time. Increasing the amount of sucrose above this will not increase the amount which will be hydrolyzed by the same quantity of sucrase preparation in that same length of time. This is evidently a maximum capacity and might be interpreted in several different ways. On the basis of the discussions in the preceding chapters, the most obvious explanation involves the view that the reaction takes place in several steps. Omitting the action of the solvent, in the first, combination of sucrase and sucrose may occur. In the second, this combination may decompose to form α-glucose and α-fructose. In carrying out the experimental determinations, it is always necessary, as already stated and as emphasized originally by O'Sullivan and Tompson,[13] to bring about equilibrium between the α- and the β-forms of the hexoses by the addition of a small quantity of alkali before determining the extent of the reaction by means of the optical rotation. If this is not done, serious errors result. The formation of this first compound between enzyme and substrate would account for the phenomenon of the saturation capacity of the sucrase observed. The practical difficulty of proving by direct experiment the existence of such a compound arises first from the fact that the enzyme is not, as yet, known in an analytically pure state, secondly, the fact that the addition compound is in (colloidal) solution, and thirdly, the continuous change due to the further reaction of the enzyme-substrate complex. Even if such a compound were precipitated, the colloidal properties of the materials make it improbable that constant stoichiometrical compositions of such addition compounds can be shown to occur ordinarily.

The fact that sucrase in the presence of sucrose in solution can be heated to a higher temperature without being inactivated than in its absence is also evidence for the existence of a compound between the two, even without considering such a compound formation to be specific for the sucrose.

The experimental results just outlined might also be interpreted on the basis of the formation of an adsorption compound between

[13] C. O'Sullivan and F. W. Tompson, *J. Chem. Soc.* 57, 834 (1890); C. S. Hudson, *Jour. Amer. Chem. Soc.* 30, 1160 (1908).

sucrase and sucrose. Plotting the velocity (concentration of sucrose divided by time for certain percentage hydrolyzed) against the concentration of sucrose gave curves essentially similar to adsorption curves such as have been obtained for example in the study of the adsorption of acetic acid by charcoal.[14] Sucrase is a colloid and this relation must be considered carefully. If adsorption is the dominating factor, then hydrolysis is assumed to take place at or near the surface of the sucrase, and the effective concentration of the sucrose or the velocity of inversion might be considered a measure of the amount of sucrose adsorbed by the sucrase. The maximum velocity attained with increase in concentration of sucrose, would represent upon this assumption, sucrase, saturated as regards adsorption.

Various mathematical expressions have been developed on the basis of the explanation of adsorption. The principle involved appears to consist essentially in replacing the concentration of the sucrose in the solution in the kinetic equation by a term involving the adsorbed sucrose. This term, according to adsorption relations, is not equal to the concentration of the sucrose in the solution, but to the numerical value of this concentration raised to some power less than unity, and very often between 0.1 and 0.5. The new kinetic equation therefore retains the same form as the simple kinetic equations given in Chapter II, except that for a monomolecular reaction, for example, the concentration of the reacting substance is replaced by the concentration raised to some power less than one and constant within certain limits and ranges of conditions for a given reaction. This constant must be determined by means of the experimental results of the reaction itself, just as the reaction velocity constant is determined.

In its simplest terms, therefore, introducing the adsorption factor into the kinetic equations involves replacing an equation containing one arbitrary constant (which is obtained from the experimental data) by an equation containing two constants (which are obtained from the experimental data). The form of the equation makes it possible to assign a probable physical significance to the second constant as well as to the reaction velocity constant.

Nelson and Griffin [15] had shown that the reaction between sucrase and sucrose solution depended on the contact of two phases, and

[14] G. C. Schmidt, *Z. physik. Chem.* **74**, 689 (1910); **77**, 641 (1911).
[15] J. M. Nelson and E. G. Griffin, *Jour. Amer. Chem. Soc.* **38**, 1109 (1916).

that the activity of the sucrase was not affected whether or not the enzyme was adsorbed to a solid, a second colloid, or distributed uniformly throughout the solution. Nelson and Vosburgh [16] showed on the basis of their results that the velocity of inversion curve, where the concentrations of sucrose were used as abscissas, had the same general shape as adsorption curves as already suggested by Henri.[17] In addition, since their results agreed with the heterogeneous reaction view and contradicted the claim that the kinetics of sucrase action conformed to the monomolecular law for homogeneous reactions, they considered that adsorption was one of the controlling factors in the kinetics of sucrase action.

An entirely different method of treating the kinetics of sucrase action was developed by Nelson and Hitchcock.[18] In place of suggesting new equations containing a greater number of constants on the basis of more or less plausible hypotheses, an empirical equation containing four constants was proposed to reproduce the results on the hydrolysis of sucrose by sucrase. The following equation with the constants evaluated by the method of least squares from the results of hydrolysis experiments was found to be suitable:

$$t = \frac{1}{n}\left[\log\frac{100}{100-p} + 0.002642p - 0.000008860p^2 - 0.0000001034p^3\right]$$

In this equation t represents the time, p the percentage inversion, and n a number whose value is directly proportional to the amounts of active sucrase present, or in other words, a relative measure of the amount of effective sucrase. The constancy of n determines whether this equation is applicable to a given experiment. It was found to hold for six yeast sucrase preparations out of eight, over a range of enzyme concentration of 12:1, at temperatures varying from 15 to 35°, and at hydrogen ion concentrations from 4.0×10^{-5} N to 3.2×10^{-7} N. The hydrolysis-time curves for the "normal" sucrases were shown to be of the same shape for the different conditions and could be made to superimpose if the time scale was multiplied by the proper constant.

The equation is therefore proposed as the criterion of normal sucrase action, and preparations which give results in disagreement

[16] J. M. Nelson and W. C. Vosburgh, *Jour. Amer. Chem. Soc. 39*, 790 (1917).
[17] V. Henri, *Z. physik. Chem. 51*, 19 (1905).
[18] J. M. Nelson, and D. I. Hitchcock, *Jour. Amer. Chem. Soc. 43*, 2632 (1921); D. I. Hitchcock, *Dissertation, Columbia University, 1922.*

with it are considered to be abnormal. This method of handling the results and treating sucrase actions should be extremely valuable. It is frankly empirical in character but in view of the uncertain nature of the ordinary theoretical deductions, furnishes a definite standard of comparison.

Nelson and Hollander[19] showed that two sucrase preparations which did not conform to the four-constant equation owed their "abnormal" behavior to lower relative stabilities, becoming inactivated gradually during the course of the reaction. Yeast gum and sodium chloride stabilized one of these abnormal preparations but not the other. Further, it was found that the pH range of optimum stability did not coincide with the pH range of optimum activity (4.5-5.5), but that greater stability was shown at pH 5.8. The sucrase was therefore less stable at the pH for optimum action than under more alkaline conditions.

A further relation may be developed with sucrase. It is of course known that various substances retard the actions of enzymes. Michaelis and Menten[20] showed that the action of sucrase was inhibited by the products of the reaction it influenced. Fructose retarded the actions much more markedly than did glucose. Furthermore, α-glucose and α-fructose are formed first and, depending upon the hydrogen ion concentration, go over into the β-forms to varying extent.[21] The affinity of the sucrase is different for the α- and β-forms of the hexoses, β-glucose inhibiting the action of the sucrase markedly, α-glucose only slightly. These and similar facts have been confirmed and extended in various directions in recent years by J. M. Nelson and his co-workers. The obvious explanation for such retardations is based upon the formation of chemical compounds of the surcase with glucose and fructose. In this way the sucrase is removed from the sphere of action and is unable to combine with sucrose in order to cause it to react further. The suggestion may be made that adsorption compounds (in contradistinction to chemical compounds) are formed by the products of the reaction with the sucrase, thus interfering with the formation of such compounds of the latter with the sucrose. The greater

[19] J. M. Nelson and F. Hollander, *J. Biol. Chem. 58,* 291 (1923). Cf. also J. M. Nelson and G. Bloomfield, *Jour. Amer. Chem. Soc. 46,* 1025 (1924).

[20] L. Michaelis and M. L. Menten, *Biochem. Z. 49,* 333 (1913).

[21] R. Kuhn, *Z. physiol. Chem. 127,* 234 (1923); H. v. Euler and K. Myrbäck, *Z. physiol. Chem. 129,* 100 (1923).

retardation shown by the fructose as well as the different actions of the α- and β-forms, point to chemical differences as the cause of the combinations. It would be of interest to obtain the results of similar careful experiments with other carbohydrates as light may possibly be thrown on the nature of the compounds with sucrase in this way.

The difference between the actions of yeast sucrase and taka sucrase found by Kuhn are of interest in this connection.[22] The following results were obtained for the action of α-glucose, of β-glucose, and of fructose on the hydrolysis of sucrose by the two enzyme preparations:

	Yeast Sucrase	*Taka Sucrase*
α-Glucose	No inhibition	Marked inhibition
β-Glucose	Marked inhibition	No inhibition
Fructose	Marked inhibition	No inhibition

Since the fructose inhibits the action of the yeast sucrase, in all probability by combining with it in such a way that the reaction with sucrose is interfered with, it is concluded that in the reaction between yeast sucrase and sucrose, the first step would be a combination of the sucrase with the fructose radical of the sucrose. Yeast sucrase would then be considered a fructo-sucrase. Similar reasoning for the taka-sucrase leads to the conclusion that it should be considered to be a gluco-sucrase. These findings, however, have not met with general acceptance.[23]

Considerable space is being devoted here to the sucrase reactions. These have been studied as extensively and as carefully as the reactions of any enzyme. The fact that the enzyme can be obtained readily, that it forms (to the eye) clear aqueous solutions (colloidal it is true), and that the chemical reaction whose velocity it influences has been carefully studied from various points of view, makes it a very satisfactory subject for study. The chemical nature of the active enzyme itself is still unknown. On the other hand, considerable information has been obtained with regard to the mechanism of the reaction. The kinetics are fairly well known. The influences of the hydrogen ion concentration, of the sucrase concentration, of the initial sucrose concentration, of the products of the reaction, of certain neutral inorganic salts and organic sub-

[22] R. Kuhn, *Z. physiol. Chem.* *129*, 57 (1923).
[23] H. v. Euler and K. Josephson, *Z. physiol. Chem.* *132*, 301 (1924).

stances, have been studied carefully. The most satisfactory general explanation of the reaction, and one that is widely accepted at the present time, involves the formation of a chemical compound of sucrase and sucrose followed by its decomposition into the hexoses.

With regard to the adsorption explanation for such enzyme actions, the same points may be developed as in the preceding chapters. The absorption view alone does not appear to offer any advantages over the view of the formation of chemical compounds except that it is less specific and does not go beyond the most superficial experimental relations observed. In fact, the application of adsorption formulas does not negative the formation of addition compounds but shows only part of a possible mechanism involved in their production. Also, the view is gaining ground, as set forth in the earlier chapters, that adsorption itself is to be explained most satisfactorily on the basis of the formation of chemical compounds.

Before leaving the subject of sucrase, some suggestive calculations of Euler [24] may be presented. On the basis of intermediate compound formation, sucrase (if present in small concentration) is taken to be combined completely with sucrose in a 4% sucrose solution. In a 0.05 N sucrose solution half the enzyme is so combined. If equal molecules of sucrase and sucrose unite, then

$$K = \frac{[\text{Intermed. Product}]}{[\text{Sucrase}] \times [\text{Sucrose}]} = 50.$$

(Michaelis and Menten [25] gave a value of 60; Euler and Laurin [26] 38). It is assumed that combination to form the intermediate compound is very rapid and that the measured rate of the reaction is given by its decomposition. In 0.2 minute, 0.05 gm. purified sucrase (If = 230) can decompose 3 gm. or 0.009 mol sucrose; at the beginning of the reaction the rate is 1×10^{-3} gm. mol per second. If the equivalent of sucrase is taken to be 4000, the concentration of the sucrase and also of the intermediate compound is 1.2×10^{-5} N, and therefore the sucrose is renewed 80 times per second on the sucrase molecule. In the acid hydrolysis of sucrose, the formation of an intermediate compound of sucrose and acid (HCl) is assumed in which the former functions toward HCl as a weak base,

[24] H. v. Euler, *Ber. 55B,* 3583 (1922); H. v. Euler and K. Josephson, *Z. physiol. Chem. 133,* 279 (1924).

[25] L. Michaelis and M. L. Menten, *Biochem. Z. 49,* 333 (1913).

[26] H. v. Euler and I. Laurin, *Z. physiol. Chem. 110,* 55 (1920).

$k_b = 10^{-20}$ approximately (in comparison with the basic properties of esters). With 0.1 N HCl, if $k = 1.5 \times 10^{-4}$, the concentration of the intermediate compound would be 10^{-8} N. Comparing the HCl and sucrase catalyses:

		Catalyses	
		Sucrase	*HCl*
Concentration	Catalyst	0.6×10^{-8} N	0.1 N
	Intermediate compound ..	0.5×10^{-8} N	10^{-8} N
Affinity constant of intermediate compound		50	10^{-6}

For the same reaction velocity, the concentration of the intermediate compound would be the same for the enzyme and acid. In the one case this is due to a small concentration of catalyst (enzyme) with a large affinity for the substrate, in the other case to a large concentration of catalyst (acid) with a small affinity for the substrate.

These calculations cannot be taken as very accurate, but only indicate orders of magnitude. They are nevertheless suggestive in that they give a possible picture of the mechanism of the reaction under different conditions.

The relations outlined with sucrase will now be applied to enzyme actions in general. The underlying theory of the mechanism of the hydrolytic action on sucrose involves the formation of an intermediate addition compound of enzyme and substrate. Following the scheme of formulating reactions given in Chapter II, the following equations may be given as representing some of the possible actions in a complex mixture such as would be present in an enzyme action.

$$\left[\begin{array}{c}\text{Enzyme}\\ \text{Substrate}\\ \text{(or Products)}\\ \text{Water}\end{array}\right] = \text{Enz.} + \text{Substr.} + \text{Water} \quad \text{(a)}$$

$$= \text{Enz.} + \text{Products} + \text{Water} \quad \text{(b)}$$

$$= \text{(Enz. Products)} + \text{Water} \quad \text{(c)}$$

$$= \text{(Enz. Substr.)} + \text{Water} \quad \text{(d)}$$

These equations do not show the actions of any other substances, and in fact give only the simplest changes involved. Starting with the substances of the right hand side of equation (a), if the products of equation (b) are obtained, the simplest case possible would be given. The enzyme represented as unchanged in composition would be considered to be the catalyst.

The first question which may be raised and which was not con-

sidered with sucrase, is whether starting with (b) the products of equation (a) would be obtained; in other words, whether enzyme actions are reversible. It has been found that a number of them are reversible, that certain enzymes increase the velocity of formation of more complex bodies from simpler ones. Questions of suitable concentration of the substances present are obviously involved and require a certain amount of experimentation to determine, but otherwise, the theoretical deduction that an enzyme, in its simplest condition of acting, increases the velocities of both reactions involved in an equilibrium has been found to hold. Further details of this reversibility will not be given but only a list of some of the enzymes which have been found to influence the synthetic changes will be presented. These include lipase,[27] emulsin,[28] trypsin,[29] pepsin,[30] kephirlactase,[31] maltase,[32] oxynitrilase,[33] and urease.[34] In some of the enzyme preparations a mixture of enzymes was present; for example, maltase from yeast extract contained at least five sucroclasts, emulsin contained at least three, etc. This served to complicate apparently some of the syntheses observed. For instance, glucose with the maltase enzyme mixture gave isomaltose in place of the expected maltose, etc. However, these relations can be

[27] J. H. Kastle and A. S. Loevenhart, *Am. Chem. J.* *24*, 491 (1900); M. Hanriot, *C. r.* *132*, 212 (1901); H. Pottevin, *Ann. Inst. Pasteur*, *20*, 901 (1906); *Bull. Soc. Chim.* *35*, 693 (1906); A. E. Taylor, *J. Biol. Chem.* *2*, 87 (1906); M. Bodenstein and W. Dietz, *Z. Elektrochem.* *12*, 605 (1906); W. Dietz, *Z. physiol. Chem.* *52*, 279 (1907); A. Welter, *Z. angew. Chem.* *24*, 385 (1911); M. Krausz, *Z. angew. Chem.* *24*, 829 (1911); U. Lombroso, *Arch. pharm. sper.* *14*, 429 (1912); K. Bournot, *Biochem. Z.* *52*, 172 (1913); H. E. Armstrong and H. W. Gosney, *Proc. Roy. Soc. London (B)* *88*, 176 (1914); A. Hamsik, *Z. physiol. Chem.* *90*, 489 (1914); L. Spiegel, *Z. physiol. Chem.* *127*, 208 (1923).

[28] O. Emmerling, *Ber.* *34*, 3811 (1901); J. H. van't Hoff Sitzungsber, Kgl. Pr. *Akad. Wiss. Berlin*, *1910*, 963; E. Bourquelot, H. Hérissey, and J. Coirre, *C. r.* *157*, 732 (1913); *J. Pharm. Chim.* (7) *8*, 441 (1913); E. Bourquelot (review), *J. Pharm. Chim.* (7) *10*, 361, 393 (1914); G. Zemplen, *Ber.* *48*, 233 (1915); E. Bourquelot and A. Aubry, *C. r.* *163*, 60 (1916), *164*, 443, 521 (1917); E. Bourquelot and M. Bridel, *C. r.* *168*, 253, 1016 (1919); *Ann. Chim. Phys.* (8) *28*, 145 (1913); W. M. Bayliss, *J. Physiol.* *46*, 237 (1913).

[29] A. E. Taylor, *J. Biol. Chem.* *3*, 87 (1907); *5*, 381 (1909).

[30] T. B. Robertson, *J. Biol. Chem.* *3*, 95 (1907); *5*, 493 (1909); T. B. Robertson and H. C. Biddle, *J. Biol. Chem.* *9*, 295 (1911); F. P. Gay and T. B. Robertson, *J. Biol. Chem.* *12*, 233 (1912).

[31] E. Fischer and E. F. Armstrong, *Ber.* *35*, 3141 (1902).

[32] A. Croft Hill, *J. Chem. Soc.* *73*, 634 (1898); *83*, 578 (1903); *Ber.* *34*, 1380 (1911); O. Emmerling, *Ber.* *34*, 600, 2206, 3801 (1901); E. F. Armstrong, *Proc. Roy. Soc. London (B)* *76*, 592 (1905); E. Bourquelot and E. Verdon, *J. Pharm. Chim.* *8*, 19 (1913).

[33] V. H. Krieble, *Jour. Amer. Chem. Soc.* *37*, 2205 (1915).

[34] H. D. Kay, *Biochem. J.* *17*, 277 (1923).

readily accounted for as being due to the mixture of enzymes present.

A question which develops directly from the idea of reversibility as dependent to a certain extent upon the concentrations of the reacting constituents has to do with the application of the kinetic equations and the limitations of the concentration terms in them. The deduction of these equations in Chapter II was based upon the law of mass action or, in practical work, the law of concentration action. As deduced, the equations hold only for dilute solutions. The limit of concentration which can be used is undoubtedly different for the components of each reaction. However, this limitation must be kept in mind especially when working with concentrated solutions. Also, substituting concentration for active mass may cause complications and errors for substances of biochemical origin, where apparently simple treatments may result in modifications of properties which involve changes in the active mass of the constituent acting which is not reflected in the concentration as ordinarily measured and used.

To return to the general equation of enzyme action which was proposed as a consequence of the relations found with sucrase and the general theory of chemical reactions outlined in Chapter II, if equations (a) and (b) alone are considered, the enzyme would act as a catalyst, being unchanged in chemical composition as a result of the reaction. Starting with the substances on the right hand side of the equation (a), the substances on the right hand side of equation (b) would be obtained. As shown, the change involves two consecutive reactions. Some evidence for the formation of the intermediate product in enzyme reactions, in addition to that given with sucrase, may now be presented. As pointed out in Chapter IV, the formation of adsorption compounds, and the greater stability toward heat of a solution of enzyme plus substrate as compared with enzyme alone, and in Chapter V. Fisher's lock-and-key theory may serve as evidence. A line of proof analogous to the last was brought forward some years ago and confirmed in various ways since.

H. D. Dakin[35] showed that a liver lipase preparation acting upon an optically inactive mixture of dextro and levo mandelic esters hydrolyzed the dextro component more rapidly than the levo. His

[35] H. D. Dakin, *J. Physiol. 30,* 253 (1904). Cf. also E. Abderhalden, H. Sickel and H. Ueda, *Fermentforsch.* 7, 91 (1923).

conclusions may be quoted: "The dextro- and laevo-components of the inactive ester first combine with the enzyme, but the latter is assumed to be an optically active asymmetric substance, so that the rates of combination of the enzyme with the d- and l-esters are different. The second stage of the reaction consists in the hydrolyzing of the complex molecules of (enzyme + ester). Since the complex molecule (enzyme + d-ester) would not be the optical opposite of (enzyme + l-ester), the rate of change in the two cases would again be different. Judging by analogy with other reactions one might anticipate that the complex molecule which is formed with the greater velocity would be more rapidly decomposed. In the present case it would appear that the dextro component of the inactive mandelic ester combines more readily with the enzyme than the laevo component does, and that the complex molecules (d-ester + enzyme) are hydrolyzed more rapidly than (l-ester + enzyme), so that if the hydrolysis be incomplete dextro-acid is found in solution and the residual ester is laevo-rotatory." The same method of reasoning may be applied to the actions of other enzymes. For example, the hydrolysis by trypsin of a number of peptides, differing only in the optical activity of the components, was found to be markedly different.[36]

In considering the velocities of the two consecutive reactions (a) and (b), ignoring any side or other reactions which may be possible and occur, if the rate of one of these reactions is extremely large as compared with the other, the observed rate of the total reaction will be that given by the slower reaction. Thus, if reaction (a) occurring in the left hand direction is extremely rapid, and the time required for the formation of the intermediate compound very small compared to the rate of its decomposition, then the observed velocity of the total reaction will be proportional to the concentration of the intermediate addition compound. On the other hand, if the rate of decomposition of the intermediate compound is very large in comparison with its rate of formation, or, in other words, if the addition compound decomposes as soon as or immediately after it is formed, then the observed rate of reaction would be proportional to the concentrations of enzyme and substrate. These relations will be referred to again in the further discussion.

If, in equation (a), the substrate is present in very great excess

[36] E. Fischer and E. Abderhalden, *Z. physiol. Chem.* *46*, 52 (1905); *51*, 264 (1907).

as compared with the enzyme, then, especially if the initial concentration of enzyme is small, a steady state may be assumed to exist in (a) in which it may be said that all of the enzyme is combined in the intermediate compound (or a constant large fraction of it is so combined). Under these conditions, if there are no side reactions or other interfering factors, the velocity of the reaction will depend upon the concentration of the intermediate addition compound, which is constant as long as sufficient excess of substrate is present, provided that the decomposition of the intermediate compound does not occur instantaneously but requires a measurable time. This means that as long as these conditions hold, the same absolute amount of intermediate compound is decomposed in each unit of time, or the same amount of substrate is decomposed, or the same amount of products formed. This relation, a maximum capacity of an enzyme preparation for hydrolyzing a substrate, no matter how much excess substrate may be present above a certain quantity, has been observed for a number of enzymes and taken to be evidence for the formation of such an intermediate addition compound. The following examples may be mentioned:

Sucrase	:	sucrose [37]
Amylase	:	starch [38]
Lactase	:	lactose [39]
Maltase	:	maltose [39]
Emulsin	:	β-glucosides [39]
Lipase	:	esters [40]
Urease	:	urea [41]

This change, when plotted, would show a linear relation between the quantity of substrate decomposed and the time, and not a logarithmic relation as required by the monomolecular reaction velocity equation.

[37] A. Brown, *J. Chem. Soc. 81*, 373 (1902); E. Duclaux, *Traité de Microbiologie, Tome II*, Diastases, Toxines, et Venins, Paris, 1899; L. Michaelis and M. L. Menten, *Biochem. Z. 49*, 333 (1913); J. M. Nelson and W. C. Vosburgh, *Jour. Amer. Chem. Soc. 39*, 790 (1917); K. G. Falk and G. McGuire, *J. Gen. Physiol. 3*, 595 (1920-21).

[38] H. T. Brown and T. A. Glendinning, *J. Chem. Soc. 81*, 388 (1902); C. L. Evans, *J. Physiol. 44*, 191 (1912).

[39] E. F. Armstrong, *Proc. Roy. Soc. London (B) 73*, 500 (1914).

[40] H. C. Bradley, *J. Biol. Chem. 8*, 251 (1910); G. Peirce, *Jour. Amer. Chem. Soc. 32*, 1517 (1910); K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc. 37*, 217 (1915).

[41] D. D. van Slyke and G. E. Cullen, *J. Biol. Chem. 19*, 141 (1914).

In a number of cases the amount of action was found to be proportional to the enzyme concentration (with large excess of substrate present).

The evidence may be considered sufficient to prove the formation of intermediate addition compounds with the enzyme in reactions whose velocities are increased by enzyme preparations.

If the substrate is not present in great excess, and if there is no steady state existing in either of the two simultaneous reactions, then the kinetics would be represented by the equations for consecutive reactions which were given in Chapter II. These equations are very complex and require further assumptions and simplification in order to be used in any given case. In view of the experimental complications involved in many enzyme actions, some of which will be taken up presently, it does not seem to be worth while to enter into the details of this question at present.

Although the relations presented appear to be clear and straightforward and to be supported satisfactorily by the experimental evidence, some work published recently by Northrop [42] indicates that perhaps the explanations advanced are not entirely complete, but that further work is required in order to clear up a number of questionable points in the mechanism of some enzyme actions.

In the first place Northrop showed that increase of the concentration of gelatin above 2% did not increase the amount digested by a given amount of trypsin. According to the views outlined above, this would indicate a maximum combination of enzyme and substrate, or saturation of the enzyme. It was found that the action of a given amount of trypsin on a mixture of gelatin and casein, even with the former in concentrations greater than 2% was equal to the sum of the two separate actions. This indicates at first sight that two different enzymes are acting, each on its substrate. However, it was also found that the presence of casein protected the gelatin-splitting power of the trypsin from heat inactivation, and the presence of gelatin protected the casein-splitting power from heat inactivation. This result negatives the view that two enzymes are present but is rather strong evidence for the fact that the same enzyme attacks both the gelatin and the casein. To sum up the conclusions reached by Northrop, if the formation of an intermediate compound is assumed, and its rate of decomposition is large in com-

[42] J. H. Northrop, *J. Gen. Physiol.* **6**, 239 (1923-24).

parison with its rate of formation, then, in order to account for the additive action on casein and gelatin, the presence of two enzymes must be accepted, while to account for the protective action of each substrate for the enzyme action of the other, the same enzyme must be assumed to attack both. If, on the other hand, the intermediate compound is assumed to decompose as soon as formed, the rate of its formation being large in comparison with the rate of its decomposition, then the additive and protective actions can be accounted for readily, but the saturation capacity of the enzyme would remain unexplained. Further study of similar enzyme actions and of the simultaneous action of inorganic catalysts on two or more reactions should aid in throwing light on these relations.

Another factor may be considered here. If a complex substrate is being decomposed, a number of products may be formed (as in the breaking down of a protein or a starch). The second stage of the reaction would then consist of a number of consecutive reactions. The extent of the changes in the substrate might then give different results depending upon whether the decomposition of the substrate or the formation of different sets of products was being studied. This was pointed out in Chapter VI, in which the results of Sherman and his co-workers were given, which showed that somewhat different results were obtained in studying the action of amylase upon starch, depending upon whether the amyloclastic (starch-splitting) or saccharogenic (sugar-forming) properties were followed. Some apparently contradictory results recorded in the literature might be accounted for in this way.

The conditions in any one series of experiments must be kept constant. In order to obtain comparable results for kinetic studies, it is advisable to work at the optimum hydrogen ion concentration of the reaction. Two investigations give some suggestive data in this connection, indicating perhaps a possible mass action effect in the enzymic hydrolysis of proteins. Frankel[43] found that when papain acted upon gelatin or egg albumin (optimum pH 5.0), if the reaction initially was more acid or more basic than the optimum, it tended gradually to approach the optimum hydrogen ion concentration. In other words, the hydrolysis may have taken place in different ways in the two cases, in the first, a greater number of amino groups than of acid groups were formed, in the second, a

[43] E. M. Frankel, *J. Biol. Chem. 31*, 201 (1917).

greater number of acid than of amino groups. If the reaction started at pH 5.0, the basic and acid groups would be formed in a ratio which kept the acidity constant. Essentially the same phenomenon was observed by Long and Hull [44] in the action of trypsin on fibrin or casein. On the other hand, this change may have been due to the properties of the aminoacid or similar substance split out by the action of the enzyme. The aminoacid may have an isoelectric point in the neighborhood of pH 5.0 and, exerting a buffer action, tend to bring the acidity nearer to that point.

The hydrolysis of urea to form ultimately ammonia and carbon dioxide has been studied quite extensively from the enzymic point of view because of the very simple chemical composition of the substrate, of the marked specificity of action of soy bean urease which for example hydrolyzes urea readily but methyl urea only slightly, and of the possibility that the reaction is one of the simplest examples of a general group of reactions which includes the hydrolysis of the proteins and similar substances.

The mechanism of the reactions involved in the hydrolysis of urea in the absence of enzymes based upon the work of E. A. Werner, mainly, was described in Chapter III. It has been found on careful study that this same general mechanism can be applied as well to the reaction when influenced by the enzyme urease. The conclusions of Werner, which were developed to a large extent as a result of the experimental studies of Fearon [45] may be best given in his own words [46]: "1. Cyanic acid has been isolated, as the silver salt, during the zymolysis of urea by urease in aqueous solution. 2. The concentration of cyanic acid was found to rise to a maximum, after which it remained constant during the greater part of the reaction. It was therefore being continually produced as fast as it was removed by hydrolysis. 3. Biuret was formed during the decomposition. This, we have seen, could only arise, in all probability, from an interaction of cyanic acid and unchanged urea. 4. Urease was found to attack urea in the presence of absolute alcohol, or at all events in a liquid containing not more than 0.82% of water. Ethyl allophanate, urethane, and biuret were the products formed,

[44] J. H. Long and M. Hull, *Jour. Amer. Chem. Soc. 39,* 1051 (1917).

[45] W. R. Fearon, *Biochem. J. 17,* 84, 800 (1923). Cf. also E. Mack and D. S. Villars, *Jour. Amer. Chem. Soc. 45,* 505 (1923).

[46] E. A. Werner, "The Chemistry of Urea." Longmans, Green and Co., London, 1923, pp. 109 seq.

thus proving the formation of cyanic acid. 5. Urease was found to be capable of combining with ammonia and of adsorbing urea. The mechanism of the reaction is explained by Fearon thus: urease condenses urea by adsorption on its surface; this is followed by the dissociation of the urea into ammonia, which combines with the enzyme, and cyanic acid which is hydrolyzed by the solvent, in the case of water. It is suggested that dissociation of urea may be brought about by (1) pressure in the adsorption area, (2) temperature of adsorption, (3) effect of an electric surface field, since urease has been found to carry an electronegative charge and to combine with ammonia.

"Apart from any theory of the mode of action of the enzyme in the first step of the change, the outstanding result of Fearon's researches proves that *urease is not directly concerned in the 'hydrolysis of urea at all.* The function of the enzyme is to bring about dissociation of urea into ammonia and cyanic acid. The hydrolysis of the latter follows as a secondary change in the presence of water. The theory that urease acts as a dissociating agent in attacking urea offers a new conception of enzyme action which it is not unlikely may be applied to other cases. We have in this theory a rational explanation of the specific action of urease. It was pointed out that methyl- and ethyl-ureas require a higher temperature than urea for their dissociation, and hence the velocity of their 'hydrolysis' when heated in the presence of acids and alkalies respectively is much slower than in the case of urea. Now, no enzyme has been found to exert its activity above 80°, whilst many are inactive at about 70°; hence if the enzyme cannot bring about the dissociation of a substituted urea below 80°, say, the latter cannot be 'hydrolyzed.' Fearon has shown that whilst methylurea is not attacked by soy bean urease up to the limit temperature, ethylurea is attacked slowly by the enzyme at 70°. This is an interesting fact in support of the new theory, since it appears to indicate that urease is capable of bringing about the dissociation of ethylurea—less stable than methylurea—just below the temperature limit of its activity.

"Quite recently Fearon has found that pure normal butylurea is attacked by soy bean urease at 45-50°. Now this is a point of further interest, since this urea, being less stable than ethylurea, is dissociated at a lower temperature, and hence is attacked by the

enzyme at a temperature below that at which it can decompose the ethyl derivative."

The action of urease in the decomposition of urea according to Fearon may be summarized as taking place in the following stages:

(1) Formation of adsorption compound, HN : C(NH$_3$)(O) (urease), in alcoholic or aqueous solution.

(2) Dissociation of this adsorption compound into ketocyanic acid and ammonia which is held in combination by the enzyme

$$HN{:}CO + NH_3\,(\text{urease})$$

(3) Hydrolysis of ketocyanic acid in aqueous solution only

$$HN{:}CO + 2\,H_2O = NH_3 + H_2CO_3 \rightarrow NH_4HCO_3.$$

(4) Combination of carbonic acid and acid carbonate with the ammonia, of the ammonia — urease adsorption compound and liberation of the enzyme

$$NH_4HCO_3 + NH_3\,(\text{urease}) \rightarrow (NH_4)_2CO_3 + (\text{urease}).$$

The cycle of the reactions then repeats itself with fresh urea. The fourth stage, the breaking down of the adsorption compound is considered by Fearon to be the true characteristic of the urea: urease system.

Some possible complicating factors in enzyme actions must now be considered. In the preliminary outline of sucrase action, it was pointed out that the products of the reaction may interfere by combining with the enzyme and so removing it from the sphere of action. This possibility is indicated in equation (c) of the general formulation. It can readily be studied by adding some of the products which would be formed in the reaction at the beginning of the action. Combination of enzyme and products has been found to occur with a number of the enzymes. In addition to sucrase to which reference has been made, the same relation was found for amylase and maltose,[47] pepsin and peptone (or albumose),[48] and doubtless others.

[47] A. Wohl and E. Glimm, *Biochem. Z.* **27**, 349 (1910); G. McGuire and K. G. Falk, *J. Gen. Physiol.* **2**, 224 (1920); O. Holmbergh, *Arkiv f. Kemi. Minerol. Geol.* **8**, *No.* **33** (1923).

[48] J. H. Northrop, *J. Gen. Physiol.* **2**, 471 (1920).

Related to these actions is the influence of inhibiting substances on enzymes, a number of which have been given in earlier chapters, and explained most satisfactorily as a combination of the inhibiting substance with the enzyme, thereby preventing the combination of the enzyme with its substrate and the consequent transformation of the latter. The antitryptic action of serum albumin for example was explained in this way.[49] This relation is mentioned here as indicating one of the possibilities of retarding action which must be watched for in studying the kinetics of enzyme actions. The question may be raised whether the anti-enzymes which are spoken of at times do not exert their influence in this way by combining with the enzyme and being of such nature themselves that they are not decomposed.

Another possible complicating factor in following the kinetics and studying the mechanism of enzyme actions has to do with the substrate. Many enzymes act upon substrates of very complex nature. It would not be surprising, therefore, if such substrates would have to be placed under definite conditions in order to obtain optimum, or perhaps even at times, marked hydrolytic actions. This question was taken up with the proteins in a number of investigations. For example, as already described, Northrop[50] considered that there is an optimum zone of hydrogen ion concentration for the combination of pepsin and protein corresponding to an optimum of digestion. He suggested that the pepsin combined largely or entirely with ionized protein. Long and Hull[51] had come to similar conclusions for the action of trypsin, suggesting that it was the combination of enzyme plus substrate rather than the enzyme alone which was affected by the reaction. Ringer[52] also has emphasized the importance of the substrate in the action of pepsin. He, however, considered that the optimum action depended upon and corresponded to a maximum swelling (hydration) of the protein.

These investigations all point to the importance of the complex substrate in enzyme actions. Chemical changes are evidently involved here as in the changes which enzymes undergo in becoming

[49] S. G. Hedin, *Z. physiol. Chem. 52,* 412 (1907); cf. also R. G. Hussey and J. H. Northrop, *J. Gen. Physiol. 5,* 335 (1922-23).

[50] J. H. Northrop, *J. Gen. Physiol. 2,* 113 (1919).

[51] J. H. Long and M. Hull, *Jour. Amer. Chem. Soc. 39,* 1051 (1917).

[52] W. E. Ringer, *Z. physiol. Chem. 95,* 195 (1915); *Kolloid. Z. 19,* 253 (1916).

inactivated. It appears to be perfectly proper therefore to say that a substrate is inactivated when, by a simple change in conditions, it is found to be no longer in a state to be acted upon by an enzyme. From this point of view, such differences would present only a phase of the general phenomenon of specificity.

There is another way in which the substrate can exert an influence. This may be illustrated by the action of a castor bean lipase preparation on a number of different esters,[53] and also its action in the presence of different alcohols on an ester.[54] The results showed that retardation on the hydrolysis of ethyl butyrate was found with methyl and ethyl alcohols, the amounts of the retardation increasing with the concentration. On the other hand, glycerin tested in the same way as the alcohols caused no retardation even up to a concentration of 25%.[55] The hydrolytic action of the lipase preparation was then tested on varying concentrations of a number of different esters. A certain complication was introduced by the fact that a mixture of ester-hydrolyzing enzymes was undoubtedly present. Starting with no ester and increasing the quantity up to molar concentration, it was found that with methyl acetate increasing actions were obtained up to 0.2-0.5 molar and then decreasing actions; with ethyl acetate, the same, beginning to decrease at a smaller concentration; with ethyl butyrate, increasing actions up to 0.2 molar reaching a value double that of the ethyl acetate, and then remaining constant for the greater concentrations (possibly because the limit of solubility of the ester had been reached); and with glyceryl triacetate, continually increasing actions with increasing concentrations, with little of the marked variations shown by the other esters.

These results justify the extension of the explanation advanced for the action of the simple alcohols suggested in Chapter IV to the action exerted by the simple esters; i.e., the ester causing a change, possibly accompanied by precipitation or coagulation of substances in the course of which the active lipase material is partially or wholly removed from the sphere of action. Methyl acetate and ethyl acetate showed least increase in activity with increasing concentration of ester and therefore the greatest inhibit-

[53] K. G. Falk, *Jour. Amer. Chem. Soc. 35*, 1904 (1913).

[54] K. G. Falk, *Jour. Amer. Chem. Soc. 35*, 616 (1913).

[55] Cf. also H. E. Armstrong and E. Ormerod, *Proc. Roy. Soc. London (B) 78*, 376 (1906).

ing action. Glyceryl triacetate showed the greatest increase in activity with increasing concentration of ester and therefore the smallest inhibiting action. These results are exactly similar to those obtained with methyl and ethyl alcohols and glycerin. That the actions are not controlled entirely by the alcohol radicals is apparent from the fact that with glyceryl triacetate even the dilute solutions did not show a proportionality between the amount of ester and the action. To continue this view further, it is probable that the fats, such as glycerides of fatty acids of high molecular weight, exert little or practically no inhibiting action. The rate of their hydrolysis would then depend upon the possibility of dissolving the fat or of bringing it in direct contact with the enzyme. The extent to which substances such as bile salts act in this way does not appear to be quite certain as yet.

These results appear to account for part, at any rate, of the specific actions of the lipase on the different esters. In these results, it must be remembered, the hydrogen ion concentration was not fixed. However, many of these and analogous experiments on specificities are incomplete in this way. The explanation and analogy given may therefore account for them. However, for more complete and satisfactory conclusions to be reached it is necessary to consider the hydrogen ion concentrations, possible salt actions, etc.

A similar, or possibly analogous, action of substrate on enzyme must be watched for in every enzyme action. The effect may be small or even absent, or again, as with lipase, it may be quite marked.

These two possible effects or actions of substrate on enzyme are typified by equation (*d*) in the general reaction. The enzyme-substrate is indicated in parentheses to show that there is no permanent compound formed and remaining as a product, but only to indicate that the substrate acts on the enzyme in some way.

The consideration of the mechanism of enzyme actions shows the difficulty of applying simple kinetic equations to the reactions. Only in special cases do the results show that certain relations hold. It may be mentioned again that Bayliss,[56] following Bredig,[57] pointed out that the best means of comparing the relative velocities,

[56] W. M. Bayliss, *Proc. Roy. Soc. London* (*B*) *84*, 87 (1911).
[57] G. Bredig, *Ergeb. Physiol. 1*, 134 (1902).

without assuming the application of some law of reaction velocity, was to use the time required to effect a definite change as a measure of the reaction velocity, a method suggested and used by O'Sullivan and Tompson in 1890 in studying the enzymic hydrolysis of sucrose.

A number of attempts have been made to derive general equations for enzyme actions involving relations similar to those given in equations (*a*), (*b*), and (*c*), of the general formulation. The developments outlined in connection with the action of sucrase in the earlier part of this chapter indicate some of the lines which have been followed. Unfortunately, in most of the attempts at the development of general equations which have been made, expressions were obtained which contained two or more constants which had to be obtained from the experimental results to which they were later applied. The expressions obviously were found to hold more satisfactorily than the more usual one-constant expression, but did not prove that the relations postulated were correct. Also, at times, new relations would be developed and conclusions drawn which involved assumptions which were sometimes stated and sometimes omitted.

At the same time, some deductions have been made and kinetic equations developed in which the complex nature of the problem was recognized and the limitations of the equations, as well as the assumptions made, clearly stated. The experimental and theoretical work of Northrop, following certain empirical conclusions developed by Schütz a number of years before, may be quoted in this connection.

Schütz [58] had found experimentally that in the action of pepsin on egg albumin, the amounts of egg albumin digested (to peptone) in a given time with different quantities of pepsin were proportional to the square roots of the concentrations of pepsin. This rule was found to hold for the first part (one-third to one-half) of the reaction by different workers. Also, the rule was found to hold for different enzymes, including lipase and diastase, under certain conditions.

Arrhenius [59] showed that in the hydrolysis of ethyl acetate present in great excess by ammonia (or ammonium hydroxide), the mathe-

[58] E. Schütz, *Z. physiol. Chem.* **9**, 577 (1885).

[59] S. A. Arrhenius, *Medd. Kong. vetsakad, Nobelinst.* (1908) **1**; "Quantitative Laws of Biological Chemistry," 1915, p. 41.

matical equation representing the change was analogous to the equation of Schütz's rule after the first moments of the reaction. The ammonium ion of the ammonium acetate formed in the reaction repressed the ionization of the ammonium hydroxide and therefore the concentration of the hydroxyl ions. The velocity would be inversely proportional to the amount of ammonium acetate formed after the first few minutes of the reaction. The following general equation was deduced:

$$A \log_e \frac{A}{A-x} - x = kqt$$

in which A represents the concentration of ammonia at the beginning of the reaction, x the quantity transformed into ammonium acetate at the time t, q, the concentration of ester, and k the reaction velocity constant. Before x becomes too large (as with the enzyme actions just mentioned) this equation reduces to the form:

$$x = \sqrt{kAqt}$$

which is an expression of Schütz's rule, and which was found to hold for the hydrolysis of ethyl acetate by ammonia under the indicated conditions.

A more exact equation along similar lines was deduced by Northrop [60] in connection with the study of the digestion of proteins by pepsin in order to represent the complete course of the reaction. The action was shown to be caused by free pepsin, and the amount of free pepsin, after the first few minutes, was found to be inversely proportional to the amount of products. The pepsin was present in solution free or in combination with the products of hydrolysis of the protein, the relative concentrations following the law of mass action. The equation of Northrop is as follows:

$$q \log_e \frac{q}{q-x} - x = kEt$$

E representing the concentration of enzyme. The equation differs from that of Arrhenius in that in the latter the substrate concentration was assumed to remain constant while the enzyme concentration was represented by the term (A-x)/x, and in the former, the substrate concentration was expressed by the term q-x, and the enzyme concentration by the term E/x. Neither the Arrhenius

[60] J. H. Northrop, *J. Gen. Physiol.* **2**, 471 (1920).

nor the Northrop equation represents the experimental facts for the first few minutes of the reaction (until the concentration of substrate decomposed is large, ten to fifteen times as great as the concentration of active pepsin). Both equations simplify to Schütz's rule for the next thirty to forty per cent of the reaction, and after that, when the substrate is no longer present in great excess and its concentration can no longer be considered constant in the mathematical formulation, the Northrop equation more nearly represents the facts.

These deductions, strictly speaking, apply only to the pepsin-protein reaction. The general method of treatment should, however, be applicable as well to other enzyme actions. A study of the kinetics of some tissue lipase actions [61] interpreted in terms of the monomolecular reaction velocity equation, Schütz's equation, and Northrop's equation, gave results similar to those obtained in the study of the protease actions. The values of k of the monomolecular velocity equation were found to decrease steadily or after a short initial period of constancy (the decrease in some cases being due to change in hydrogen ion concentration, in other cases, where this remained constant, to the increasing concentration of the products of the reaction); the values of k_s of Schütz's equations were found to increase for the first part of the reaction then to remain constant for various periods of time, and then in some cases to decrease again (the initial increases being due to the insufficiency of the reaction products, while after a certain concentration of these, the velocity became inversely proportional to their concentration k_s remaining constant, and when the change in substrate concentration had become large, the volumes of k_s decreasing since Schütz's equation contains no term to include such a change); the values of k_n of Northrop's equation following initial irregularities or increases, were found to give constant values over greater or smaller ranges, followed in some cases by decreases. In general, it may be stated that although the application of Northrop's equation to these experimental results did not give better constants than Schütz's equation, the theoretical bases underlying his deductions are more satisfactory.

In a series of papers published recently,[62] Northrop studied the

[61] K. Sugiura, H. M. Noyes, and K. G. Falk, *J. Biol. Chem. 56*, 903 (1923).
[62] J. H. Northrop, *J. Gen. Physiol. 6*, 417, 429, 439 (1923-24).

kinetics of trypsin action more carefully, and by modifying the experimental conditions so that various factors were successively eliminated, showed that the course of the reaction could be formulated on certain comparatively simple assumptions. One of the interesting points which was developed in the course of this study was "that the various equations that have been proposed to account for the enzyme reactions on the basis of a compound between the enzyme and substrate could be applied equally well on the basis of a compound between water and the substrate which is attacked by the enzyme." It would lead too far to enter into the details of the deductions at the present time. It is to be hoped that the methods of study used and the equations developed will be applied to other enzyme reactions in order to obtain conclusions of general validity and applicability.

VIII.—Uses and Applications of Enzymes

In this chapter an attempt will be made to indicate some of the circumstances under which enzymes and enzyme actions touch upon the problems and manifestations of things met with in the ordinary course of affairs. The utilitarian aspects rather than the purely scientific (or, as some would have it, non-utilitarian) side of enzyme actions will be emphasized. At the same time it will be seen that enzyme actions show clearly the close relationship which exists between the scientific and the practical aspects of chemical phenomena.

Enzymes have been defined as catalysts produced by living matter. It is difficult to give a satisfactory rigid definition of living matter, but the evidence available at the present time indicates that the chemical reactions which take place during life processes obey the same laws as chemical reactions unconnected with such processes. The evidence is only fragmentary at the present time, it is true, but this may be due to the complexity of the substances involved and the lack of accurate knowledge concerning their chemical nature. The complexity of these substances also involves the view that they may react in a number of different ways. Also, none, or very few, of them may be said to be (thermodynamically) stable. That is to say, given sufficient time, practically all of them would decompose to give finally a limited number of stable products in appreciable quantity, together with traces of a greater number of products.

The chemical reactions which take place in living matter must be influenced in such a way that the products essential to the life process are formed, if life is to continue. The actions of enzymes would therefore consist in favoring or increasing the velocities of those reactions which are required in the life process and making possible its continuance. On the basis of the general theory of chemical reactions referred to in the earlier chapters of this book, this would mean that the complex addition or intermediate com-

pound formed by the reacting substances plus enzyme catalyst reacts in certain more or less definite ways.[1] In this way certain products would be formed with velocities sufficiently great to make other possible products, as well as products obtainable by the decomposition of the addition compound without enzyme catalyst, ordinarily negligible in quantity. The function of the enzyme is, therefore, to favor the production in living matter of certain products which might otherwise be formed in only small amounts. The question may be raised whether the enzyme catalyst can start a reaction or only modify the velocity of a reaction which can take place in its absence but much more slowly. This question has been much discussed. According to certain thermodynamical considerations, the catalyst only modifies the velocity of a reaction; but practically speaking, a catalyst may increase the velocity of a reaction which proceeds almost infinitely slowly in its absence, sufficiently to make the reaction of practical use.

It has already been indicated that enzyme actions are present in all living matter and are connected with the chemical changes occurring there. This includes all the phenomena accompanying growth, such as all metabolic and catabolic changes. Enzymes are only concerned with systems in process of chemical change. Evidence has been accumulating to show that an enzyme in a definite preparation is capable of increasing the velocity of a more or less definite reaction. This reaction may refer to a change taking place in one substance only, or it may refer to analogous changes occurring with a group of related substances (such as the hydrolysis of esters). If a given preparation can increase the velocities of reaction of two different groups of substances, then it is customary to assign two distinct enzymic properties or enzymes, one for each group of reactions, to the preparation. This property of specificity spoken of in Chapter V will be referred to again in Chapter X. It is of interest and importance in this connection as it is one of the most significant of the enzyme properties and is made use of in almost every connection in which enzymes and enzyme reactions are employed.

The uses and applications of enzymes may be divided roughly into the following groups:

[1] Cf. K. G. Falk, "Catalytic Action," Chapter VII, "A Chemical Interpretation of Life Processes."

Industrial applications of enzymes.
Enzymes of metabolism and catabolism.
Enzymes in plant growth.
Bacterial enzymes.
Enzymes in laboratory work.

This list does not pretend in any way to be complete. It will serve to indicate the manifold possibilities of the utilitarian side of enzyme actions. Obviously it would lead too far to enter into a discussion of any one of these groups to any extent here. Monographs might be written on any one of them. At the same time it is true that, in the past, the work with them has been descriptive to a great extent. The enzymes which have been studied extensively, such as some of those involved in the chemical reactions of metabolism, have been included in the earlier chapters.

With regard to the industrial applications of enzymes, the fermentation industries represent perhaps the most important group at the present time. A number of the problems connected with alcoholic fermentation have been studied by A. Harden, and described in some detail in his "Alcoholic Fermentation."[2] The discovery by Buchner[3] that the liquid obtained from yeast cells by the use of high pressure after grinding with sand, in the complete absence of cells was capable of forming carbon dioxide and alcohol from sugars, showed that the action was due to an enzyme which he called zymase and was not directly dependent upon the life process. Reference may be made to the monograph of Harden for the various directions these studies have taken. Only a few points can be mentioned here. The yeast juice contains a mixture of enzymes which can give rise to a number of different products. The zymase which converts hexoses into alcohol and carbon dioxide is of course the most important enzyme or group of enzymes. Pasteur[4] showed in 1860 that glycerin was formed by the action of yeast on invert sugar. In view of the glycerin shortage in recent years, attempts were made to apply the fermentation process to this purpose. This was successfully accomplished[5] and glycerin will undoubtedly be produced

[2] Third Edition, Monographs on Biochemistry, Longmans, Green & Co., London, 1923.

[3] E. Buchner, *Ber. 30*, 117, 1110 (1897).

[4] L. Pasteur, *Ann. Chim. Phys.* (3) *58*, 347 (1860); cf. also E. Buchner and J. Meisenheimer, *Ber. 39*, 3201 (1906).

[5] W. Connstein and K. Lüdecke, *Ber. 52*, 1385 (1919); C. Neuberg and E. Reinfurth, *Biochem. Z. 89*, 365 (1918); *92*, 234 (1918); *Ber. 52B*, 1677 (1919); E. Zerner,

in this way to a certain, doubtless increasing, extent in the future. The principle involved may be outlined as follows: [6] Glycerin is formed to a small extent, perhaps 3%, from sugar in the ordinary yeast fermentation. If the mixture is made more alkaline by the addition of various substances such as sodium bicarbonate, disodium phosphate, etc., the amount of glycerin is increased up to 12-13% of the sugar decomposed. On the other hand, this more alkaline mixture is a good breeding medium for acid forming bacteria which would pollute the glycerin. The addition of a sulfite, either sodium or calcium, acts as poison for the lactic acid bacteria and others, but does not affect the yeast cells and keeps the mixture sufficiently alkaline. The yield of glycerin under these conditions is 23-36.7% of the sugar. Very nearly an equivalent amount of acetaldehyde is produced, the reaction equation apparently being as follows:

$$C_6H_{12}O_6 = C_3H_8O_3 + C_2H_4O + CO_2.$$

The acetaldehyde combines with the sulfite and can be recovered from this. The increase in sulfite concentration decreases the amounts of alcohol and carbon dioxide which are formed as in the ordinary alcoholic fermentation, until with a suitable concentration, this reaction becomes of minor importance.

The outlines given in Chapter III for the decomposition of glucose under various conditions represent some of the views which have been proposed and which appear to be most acceptable at the present time. At the same time, their incompleteness was pointed out. It is obvious that the changes which occur in fermentation processes are equally complicated. It has been possible to modify conditions so that certain products are obtained in greater yields as shown in the case of glycerin, but the knowledge of the mechanisms of the reactions is unfortunately still incomplete. This does not mean that the experimental studies have failed to add to the useful information available for such reactions. On the contrary, much valuable material has been obtained and many details have been elucidated, but a general theory which would include the various possibilities of change is still lacking. No attempt will therefore

Ber. 53B, 325 (1920); cf. also A. R. Ling, *J. Soc. Chem. Ind. 38*, 175R (1919), for process developed by J. R. Eoff, W. V. Linder and G. F. Beyer for the Treasury Department of the United States. The subject was also reviewed and many references given by K. Schweizer, *Chemie et Industrie, 6*, 149 (1921).

[6] Cf. Chapter III.

be made here to enter into the details of these studies further than has been done. It will be necessary, however, to refer to the work of two of the foremost leaders in this field at present who have added greatly to the study of fermentation actions, and to whose publications reference may be made for the details of their results. The work of Carl Neuberg has been appearing for a number of years and has aided in clearing up a number of the separate actions involved in the various aspects of fermentation.[7] In addition must be mentioned in this connection the studies of H. v. Euler and his associates[8] of some of the reactions included in the yeast fermentations more especially from the quantitative physical-chemical side.

Another great industry in which fermentation processes play a very important part is bread-making. Two recent significant studies[9] on the enzyme actions involved here may be referred to in this connection. The problem of bread making is evidently a complex one involving enzymes which hydrolyze starch in the first place, and then those which produce carbon dioxide and other products from the simpler carbohydrates originally present or formed in the process. The properties of the protein such as the gluten content necessary for the tenacious elastic framework for the retention of the gas produced, the addition of various electrolytes to aid the action of the amylase and the yeast, the most favorable hydrogen ion concentrations for the various actions and properties of the materials, the nature of the carbohydrates present initially, etc., indicate some of the additional factors involved. At the same time, the study of the enzyme actions as such, have already aided in throwing light on this important process, and it is to be expected that more valuable information will soon be available in this field.

The observations by Harden[10] that zymase and dried yeast which

[7] A recent short summary of Neuberg's work was given by W. Fuchs, *Samml. chem. u. chem.-tech. Vorträge 27*, 1 (1922); cf. also C. Neuberg, *Ber. 55B*, 3624 (1922); and C. Neuberg and J. Hirsch, *Ergebnisse der Physiol. 21*, II, 400 (1923).

[8] Cf. for example, the review by E. Hägglund, *Samml. chem. u. chem.-tech. Vorträge, 21*, 1 (1914); H. v. Euler and K. Myrbäck, *Z. physiol. Chem. 131*, 179 (1923).

[9] These studies were carried out under the supervision of the American Institute of Baking and published as Bulletins 8 and 9 of the Institute in August, 1922: L. A. Rumsey, "The Diastatic Enzymes of Wheat Flour and Their Relation to Flour Strength." F. A. Collatz, "Flour Strength as Influenced by the Addition of Diastatic Ferments."

[10] A. Harden, *Biochem. J. 11*, 64 (1917).

have been inactivated by washing can be activated again by the addition of potassium phosphate and a pyruvate or acetaldehyde, and also that a specific difference in relation to alcoholic fermentation exists between sodium on the one hand and potassium and ammonium on the other, involve interesting possibilities.

Among the other products obtained in fermentation by yeast are succinic acid, acetic acid, formic acid, esters, fusel oil, etc. Fusel oil consists of a mixture of some of the higher alcohols and probably aldehydes. F. Ehrlich [11] showed that these alcohols were formed from aminoacids present according to the following equation:

$$RCHNH_2CO_2H + H_2O = RCH_2OH + CO_2 + NH_3.$$

Ehrlich was not prepared to decide whether the fusel oil formation was a purely enzymic action or whether it was also connected with the life process of the yeast.

The work of Dernby [12] on the autolysis of yeast has been referred to in another connection but is of special significance here. Dernby studied optimum hydrogen ion concentrations for the pepsin, tryptase, and ereptase of yeast, and the changes in action with changes in acidity. The optimum pH values found were 4 for pepsin, 7.0 for tryptase, 7.8 for ereptase. Plotting the curves for amounts of actions and different pH values for the three enzymes, in order to obtain the maximum autolysis at a constant hydrogen ion concentration, he showed that the value for pH should be 6, between the optima for pepsin and the other enzymes where all can exert their actions.

Dernby's work on the autolysis of animal tissues gave similar results.[13] For example, it explains the fact that a piece of tissue does not autolyze in alkaline solution, but if placed in acid first (for pepsin action) and then in alkali (for trypsin and erepsin action) rapid autolysis occurs.

The use of various amylase preparations industrially is widespread. It is only necessary here to refer to takadiastase, pancreatin, and malt diastase.

The production in recent years of acetone by fermentation is of

[11] Cf. F. Ehrlich, *Biochem. Z.* **2**, 52 (1906), for a review and summary of this work.

[12] K. G. Dernby, *Biochem. Z.* **81**, 109 (1917).

[13] K. G. Dernby, *J. Biol. Chem.* **35**, 179 (1918).

interest as showing the industrial possibility of such a reaction[14] even if it may not be able to compete at the present time under normal conditions with the older processes. Two investigations taking up the study of this reaction were published recently. The biochemistry of *Bacillus Acetoethylicum* with reference to the formation of acetone from starch or glucose was studied.[15] The optimum condition of the medium for growth was found to be pH 8.0-9.0; for fermentation 6.0-8.0. A number of different sugars were found to be fermentable using calcium carbonate, and peptone (as source for nitrogen), 43° was found to be the most satisfactory temperature. Formic acid and ethyl, propyl, and butyl alcohols were also formed. The general conditions for carrying out the process so that acetone and ethyl alcohol were the main products and a semicontinuous method for carrying on the fermentation were described.[16] Recently, a similar study of the acetone and butyl alcohol fermentation of starch by *Bacillus Granulobacter Pectinovorum* (which was used commercially) was published.[17]

An interesting investigation on the acid fermentation of xylose by bacteria found in fresh silage, sauerkraut, manure, and in certain soils was published recently.[18] The main products obtained were acetic acid and racemic lactic acid in a proportion of 43 to 57 parts by weight. The best conditions for this action were determined.

Additional studies of similar nature might be given but would serve no useful purpose here. A comparison of the conditions used and the results obtained in various fermentations undoubtedly would give conclusions of importance and value in classifying and carrying out the desired chemical reactions.

It does not appear necessary to enter further into the indus-

[14] The following articles described micro-organisms which can yield more or less acetone: E. Kayser, *Ann. Inst. Pasteur, 8,* 737 (1894); L. Bréaudat, *Ann. Inst. Pasteur, 20,* 874 (1906); F. Schardinger, *Wien. klin. Woch. 17,* 207 (1904); *Centr. Bakteriol. 2te Abt. 14,* 772 (1905). Two processes have been used on a commercial scale: F. Bayer and Co. D. R. P. 283,107, July, 1913: 291,162, Jan., 1914; Brit. Pat. No. 14371, June, 1914; K. Delbrück and K. Meisenburg, U. S. Pat. 1,169,321; A. Fernbach and E. H. Strange, U. S. Pat. 1,044,368; 1,044,446; 1,044,447.

[15] J. H. Northrop, L. H. Ashe, and J. K. Senior, *J. Biol. Chem. 39,* 1 (1919). W. H. Peterson, E. B. Fred and J. H. Verhulst, *J. Ind. Eng. Chem. 13,* 757 (1921).

[16] J. H. Northrop, L. H. Ashe, and R. R. Morgan, *J. Ind. Eng. Chem. 11,* 723 (1919).

[17] H. B. Speakman, *J. Biol. Chem. 41,* 319 (1920).

[18] E. B. Fred, W. H. Peterson, and A. Davenport, *J. Biol. Chem. 39,* 347 (1919).

trial applications of enzymes. Reference may be made to the larger volumes on enzyme actions such as the treatise on "Biochemical Catalysts in Life and Industry,"[19] which gives a satisfactory and complete account of proteoclastic enzymes.

Some general principles involved in these actions, may, however, be stated. The advantages in the industrial use of enzyme actions may include the comparatively low temperatures at which the reactions take place, the greater or less specificity of the actions making it possible to control the products, and the probable low cost of the materials used. The disadvantage is the length of time which may be needed to carry out the reaction. This advantage can in part be met by a study of the optimum conditions of the reaction in question, application of such principles as the law of mass action as involved in the kinetics of the reaction, and a search for substances giving increased velocities.

Another possible application of enzyme actions in industry involves synthesis in place of decomposition. This possibility does not appear to have been considered to any extent as yet. Because of the comparatively small amount of data available in this field, it would be necessary to carry on considerable pioneering work before any statement of its probable success could be ventured.

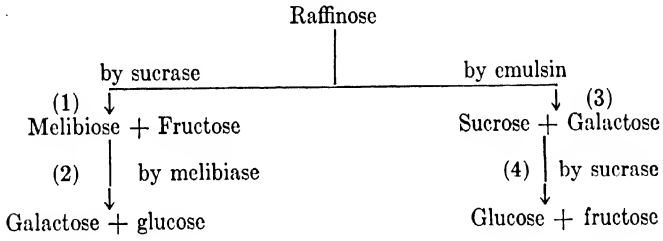
It would also appear that a careful study of the oxidizing enzymes might show some which would be of practical use.

The use of enzymes as reagents in laboratory work has been begun and promises to be of considerable value. The most striking example of their usefulness in this way is to be found with the enzyme urease. The specificity of the soy bean urease had been pointed out clearly by Armstrong and Horton.[20] For example, it was shown to hydrolyze urea and not substituted ureas such as methylurea. The accurate estimation of urea in animal fluids such as blood and urine had always been a most troublesome operation. Marshall[21] showed that the use of soy bean urease overcame all the obstacles in a very simple manner; that after the removal of the ammonia which may be present initially, addition of ground soy beans or an extract of soy beans hydrolyzed the urea quantitatively so that the determination of the ammonia formed from the urea

[19] By Jean Effront, translated by S. C. Prescott, 1917.
[20] H. E. Armstrong and E. Horton, *Proc. Roy. Soc. London* (*B*) *85*, 109 (1912).
[21] E. K. Marshall, Jr., *J. Biol. Chem. 17*, 351 (1914).

either by titration with the use of a suitable indicator or by an aeration procedure, was a simple matter. Following Marshall, a number of investigators improved the carrying out of the method in minor details. At the present time, with the use of the enzyme urease, the determination of urea has become an operation which can be carried out comparatively simply and with a satisfactory degree of accuracy.

The use of certain of the sugar hydrolyzing enzymes has been suggested and adopted in analytical work. Kjeldahl [22] and also O'Sullivan and O'Sullivan and Tompson [23] proposed to use sucrase for the determination of sucrose and Ling and Baker [24] as well as Ogilvie [25] used it for sucrose in cane and beet products. The further development has made it possible to estimate the trisaccharide raffinose by the suitable choice of enzyme materials. The method was developed by Hudson [26] and was outlined as follows:



Raffinose on treatment with mineral acids yields equal molecules of glucose, fructose, and galactose. With sucrase alone it yields melibiose and fructose; with emulsin alone, sucrose and galactose. From top yeast an extract rich in sucrase but containing no melibiase may be prepared, from bottom fermentation yeast, an extract may be prepared containing both enzymes. In carrying out an estimation of raffinose,[27] top yeast extract (sucrase) was used first. This inverted all the sucrose present and converted the raffinose into melibiose and fructose (reaction (1)). Using bottom yeast

[22] J. Kjeldahl, *Compt. rend. Carlsberg Laboratoire, Copenhagen, 1,* 189 (1881).
[23] C. O'Sullivan, *J. Chem. Soc. 49,* 58 (1886); C. O'Sullivan and F. W. Tompson, *Ibid. 59,* 46 (1891).
[24] A. R. Ling and J. L. Baker, *J. Soc. Chem. Ind. 17,* 111 (1898).
[25] J. P. Ogilvie, *J. Soc. Chem. Ind. 30,* 62 (1911); *Int. Sugar J. 14,* 89 (1912).
[26] C. S. Hudson, *Jour. Amer. Chem. Soc. 36,* 1566 (1914).
[27] C. S. Hudson and T. S. Harding, *Jour. Amer. Chem. Soc. 37,* 2193 (1915).

extract then, the change in rotation showed the hydrolysis of the melibiose (reaction (2)) and permitted of the calculation of the raffinose. If melibiose were present originally it would interfere with the determination, but in some cases this can be corrected with the original solution because melibiose reduces Fehling solution and raffinose does not.

Parenthetically it may be remarked that this scheme of analysis is similar to that used with synthetic, optically active tri- and tetrapeptides with different trypsins to find the point of attack by the enzyme by following the change in rotation.[28] The following example may be given:

$$\overbrace{\underbrace{\text{l-leucyl-glycyl-d-alanine}}_{+\,85^\circ}}^{+\,20^\circ}$$

$$\underbrace{}_{+\,10^\circ}\ \underbrace{}_{0^\circ}\ \underbrace{}_{+\,2.4^\circ}\qquad -\,50^\circ$$

Following the change in rotation on enzymic hydrolysis should show between which groupings hydrolysis occurred if the reaction took place in steps. With pancreatic extract and intestinal extract, rotation increased first to about 40° (or l-leucyl-glycine and d-alanine were formed); later the rotation decreased (hydrolysis of l-leucyl-glycine). Glycyl-d-alanine apparently was not formed at all with these reagents (if enzyme preparations may be so termed). With yeast extract, on the other hand, rotation decreased, showing that l-leucine was first separated. A number of similar examples were given, but not sufficient to enable generalizations to be drawn.

In any event, these two series of reactions with sucroclastic and proteoclastic enzymes indicate interesting and perhaps useful applications of enzyme actions.

It is possible to use certain enzymes as reagents for testing or individual substances. Although the relations between the enzymes hydrolyzing α- and β-glucosides are not altogether clear, the following behaviors may be given tentatively as indicating possibilities of actions, if not satisfactory tests. The fact that emulsin (ob-

[28] E. Abderhalden and A. H. Koelker, *Z. physiol. Chem.* *51*, 294 (1907); *54*, 363 (1908); *55*, 416 (1908).

tained perhaps best from sweet almonds) hydrolyzes β-glucosides and not α-, and that maltase hydrolyzes α-glucosides and not β-, makes it possible with these enzyme preparations to determine, whether in a doubtful case, an α- or a β-glucoside is present. A limitation is involved here in that only the dextro forms are hydrolyzed by these enzymes, not the levo. Also in a recent investigation [29] it appeared probable that potato juice contained in the neighborhood of one per cent. sucrose (or possibly raffinose), since it was acted on in this proportion by a yeast sucrase preparation which did not hydrolyze maltose.

These examples show the lines which laboratory uses of enzyme preparations have followed in recent years, and make it evident that considerable possibilities for further development exist here.

[29] G. McGuire and K. G. Falk, *J. Gen. Physiol.* 2, 215 (1920).

IX.—Enzyme Actions of Tissues and Tumors

The importance of enzyme actions for the chemical reactions which occur in life processes is frequently emphasized. Unquestionably, there is a connection between such chemical reactions, specific as all chemical reactions have been found to be, or as it is perhaps more customary to say in this connection, characteristic for a given animal tissue or vegetable material, and the enzymes which are found to be present. Possibly, and even probably, the characters of the chemical reactions depend upon the natures of the enzymes; at the very least there is an interdependence between the two.

A number of investigations published at various times have dealt with the enzyme actions obtained with various animal tissues and plant and vegetable extracts. In general terms, these studies have shown differences, more or less pronounced, in enzyme actions, of the various materials. These studies, for one reason or another, have, as a rule, been limited in scope, and, unfortunately, because of different conditions of working, the results obtained are frequently difficult to compare. At the same time, much useful and valuable material has been secured.

The results to be presented in this chapter were obtained in an attempt to go farther along the general lines indicated and to study the relations more systematically and under comparable conditions. The investigation is still in progress so that some of the lines of evidence which will be presented may perhaps be incomplete. No attempt will be made to summarize the work of others along similar lines. The present investigation includes many of the results obtained by others, so that references to the older work may suffice for the purpose in view.[1] Some of the experimental results given in Chapter VI bear upon certain phases of this problem.

[1] M. Hanriot, *Compt. rend. soc. biol.* **49**, 377 (1897); J. H. Kastle and A. S. Loevenhart, *Am. Chem. Jour.* **24**, 491 (1900); A. S. Loevenhart and G. Peirce, *J. Biol. Chem.* **2**, 397 (1906-07); A. S. Loevenhart, *J. Biol. Chem.* **2**, 427 (1906-07); M. Chanoz and M. Doyon, *J. physiol. et path. gén.* **2**, 695 (1900); E. Abderhalden and

The enzyme actions of a definite material represent certain properties of that material, just as the content in certain atoms and groups as shown by chemical analyses, and the physical behaviors as found for example in the refraction and dispersion as well as absorption of light waves of certain frequencies, are due to the composition, arrangement, and structure of the component parts of a given substance. In this sense, and in its simplest terms, enzymes would be considered to be reagents which serve to identify certain properties of the materials under investigation. This view of the problem may be of interest in various ways. For example, it may furnish a new method for the study of substances occurring in living matter and help to differentiate such substances and functions either alone or in mixtures. The difficulty frequently of such differentiation on the basis of the chemical analyses, either ultimate or proximate, is well known and need not be elaborated in this connection.

While this point of view may be considered to be true for the results to be presented in this chapter, it is probable that the enzyme actions represent more than a convenient chemical property possibly useful for distinguishing various preparations from different sources. These enzyme actions may be more directly involved in the chemical changes which are necessary in the life processes, and consequently of greater significance in their interpretation.

The present investigation involves the study of certain enzyme actions of materials obtained in normal and abnormal growth, animal tissues and tumors from various sources. A systematic study of the enzyme actions of animal tissues and tumors must be based upon a suitable choice of materials and actions in order that the results may be interpreted in a definite manner. The choice of the enzyme actions to be studied was controlled by the following conditions:—*The chemical change to be as definite as possible. The chemical manipulation to be as simple as possible,* since, in view of the nature of the materials, it was necessary to carry out a large

Y. Teruuchi, *Z. physiol. Chem.* *47*, 466 (1906); E. Abderhalden and A. Fodor, *Z. physiol. Chem.* *47*, 220 (1913); E. Abderhalden and G. Ewald, *Z. physiol. Chem.* *91*, 86 (1914); E. Abderhalden, G. Ewald, Ishiguro, and R. Watanabe, *Z. physiol. Chem.* *91*, 96 (1914); H. E. Armstrong, *Proc. Roy. Soc. London* (*B*) *76*, 606 (1905); H. E. Armstrong and E. Ormerod, *Proc. Roy. Soc. London* (*B*) *78*, 376 (1906); K. G. Falk and K. Suglura, *Jour. Amer. Chem. Soc.* *37*, 217 (1915); K. G. Falk, *Jour. Amer. Chem. Soc.* *36*, 1047 (1914); and especially the summaries given by H. v. Euler, "Chemie der Enzyme."

number of determinations within a relatively short space of time. *The possibility of varying the substrate in a more or less continuous, progressive, or systematic manner,* since it is to be expected that certain of the tissues and tumors would show small differences in actions.

The conditions imposed limited the study, in the main, to the ester-hydrolyzing enzymes of the materials, and a comparison of the actions of the tissues and tumors on a number of different esters. Two protein preparations, a peptone, and casein, were studied in a number of cases for the purpose of determining whether the protease actions paralleled the lipase actions. The results of these protease actions cannot be interpreted as simply as the lipase actions, because of the chemical complexities of the substrates.

With reference to the enzyme material, it may be pointed out that tumors of human origin are obtainable with difficulty in forms suitable for continuous laboratory study. It was necessary, therefore, to use material from a different source for the initial study. The Flexner-Jobling rat carcinoma, available in quantity, and of more or less constant properties, was used for the determination of the fundamental characteristics of the enzyme actions of a definite tumor type. The enzyme actions of the various tissues of the rat were studied similarly. The results so obtained will be presented in some detail, since they illustrate the principles involved in the method of study, its general applicability, as well as a series of fundamental underlying data which will be useful for comparative purposes. Following these, the results obtained in the study of the corresponding tissues of other animals, as well as of some human tissues will be presented, and then the results of similar studies on various groups of tumors of human origin will be shown.

The experimental methods which were used will be described briefly first.

In deciding which esters were to be used as substrates for the lipase or ester-hydrolyzing actions, it was evident that it would be difficult to obtain reproducible conditions and comparable results with insoluble esters such as the natural fats. The formation of emulsions, the presence of substances to stabilize such emulsions, and the continuous agitation of the mixture seem necessary conditions for working with these. Results will be given for the following esters: Methyl acetate, ethyl acetate, isobutyl acetate, phenyl

acetate, benzyl acetate, glyceryl triacetate, methyl butyrate, ethyl butyrate, methyl benzoate, and ethyl benzoate. Three pairs of isomeric esters are included in the list. 3.4 milli-equivalents (an arbitrary, but convenient, quantity) of ester were used in each experiment. Aside from gross impurities, which were readily removable, the impurities of the esters were tested by incubation with water at 38° for 22 hours. Negligible amounts of acid are produced, between 0.004 and 0.012 milli-equivalents as judged by titration with 0.1 N alkali.

For the protease actions, a peptone preparation and a casein preparation (purified according to Hammarsten), 0.1 gm. of each in each test, were used.

The enzyme material was obtained from albino rats, both male and female, fed on white bread soaked in whole milk, fresh cabbage or carrots, and tap water *ad libitum*. The tumor material was obtained as a result of inoculating with the Flexner-Jobling rat carcinoma in the neighborhood of the right axilla in the usual way [2] and permitting growth for from 3 to 4 weeks. The neoplasm was removed from the animal after killing it with ether. The non-neoplastic tissue was removed, the tumor material cut into small pieces or macerated with sand, and water added for extraction. The rat tissues were obtained either from normal rats or tumor-bearing rats as soon after killing with ether as possible. They were ground as fine as possible and extracted with water. The quantity of water added in each case depended upon the approximate concentration of material to be tested. Tests showed that water extraction gave very nearly the same results as extraction with physiological salt solution, so that only the former was used. Toluene was added at once. The mixtures after standing overnight were filtered through paper. Portions of 5 cc. each of the filtrates were diluted with water to 15 cc. and tested. In a number of cases, portions of the solid residues after filtration were weighed out, 15 cc. of water added, and tested similarly.

The tissues from both normal rats and tumor-bearing rats were tested because of the possibility of the presence of the tumor influencing the actions of the tissues. Such an influence was observed only with the liver and may well have been due to metastases, as will be shown later.

[2] K. Sugiura and S. R. Benedict, *J. Cancer Research*, 5, 373 (1920).

The material for each series of experiments was obtained from six to twelve rats.

The amounts of lipase actions were determined by titration with 0.1 N sodium hydroxide solution, with phenolphthalein as indicator. For the protease actions, the formal method with phenolphthalein and 0.1 N sodium hydroxide solution was used. Previous work [3] had shown that the formol and Van Slyke methods gave similar results. Corrections were introduced in every case for the enzyme material and substrate (ester or protein) blanks. Toluene was present throughout the experiments. Each test was made in duplicate.

As stated, the volumes of the solutions tested were 15 cc. The experiments were run at 38° for 22 hours. This time was chosen as most suitable as a result of the experiments on the kinetics of the actions.[4] For most of the materials the rapid hydrolyses had ceased at this time, but the actions had in no way approached completion.

The solutions were all brought to pH 7.0 initially. Because of the acid production in the ester hydrolyses, they dropped to pH 5.0 to 5.5 comparatively rapidly (much more rapidly in some cases than in others) and then remained fairly constant. Experiments carried out with the mixtures at pH 5.0 initially gave the same relative actions on the different esters as when started at pH 7.0, but smaller absolute actions. In order to obtain more satisfactory comparisons, therefore, all the actions which will be shown were obtained with the mixtures initially at pH 7.0.

It is obvious, of course, that a truer comparison of the actions would be obtained if the times for equivalent actions were taken in place of the amounts of action in equal times. Practical difficulties, due in part to the nature of the experiments, and in part to the uncertain application of the kinetics of the different actions, made it advisable to use the theoretically less accurate method of determining and presenting the results.

Two methods of presenting the results are available. In the first instance they may be given as the absolute actions of each preparation or material on the various esters. Secondly, the results for the actions of any one enzyme preparation on a number of esters

[3] K. G. Falk, H. M. Noyes and K. Suglura, *J. Biol. Chem.* *53*, 75 (1922).
[4] K. Suglura, H. M. Noyes, and K. G. Falk, *J. Biol. Chem.* *56*, 903 (1923).

may be given as relative actions; that is, denoting the greatest action by 100, and calculating the actions on the remaining esters in that series in terms of this. It will be seen that the second method of presentation is perhaps the more significant, although for a satisfactory understanding of the relations, both methods must be employed.

In Table I are shown the absolute amounts of esters hydrolyzed by the tumor and various tissues of the rat in some of the experiments. These were selected in order to indicate the magnitudes of the actions for various concentrations of the enzyme materials. In some cases the amounts of actions for the same concentration of a given tissue in different series differed considerably. Such variations in absolute actions are unavoidable when dealing with living matter.

The data in the table are self-explanatory. In Column 1, the letters RT with the number of the experiment signify that the rats from which the indicated tissue was obtained were tumor-bearing.

The results show also the relative accuracy obtainable in the different series and within any one series. For example, a comparison of two absolute actions of 0.3 each or less signifies little as to their relative actions in a series although a comparison of such actions with a much larger absolute action would be of importance.

TABLE I.

Hydrolyzing Actions in Tenths of Milli-Equivalents of Acid Produced by Rat Tissues and Carcinoma on the Indicated Esters.

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	$Gl(OAc)_3$	MeOBu	$PhCH_2OAc$	ErOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
				Flexner-Jobling rat carcinoma extracts.							
	mg.										
97	8.9	1.86	1.18	0.37	0.28	0.23	0.24	0.31	0.00	0.00	0.30
100	17.8	2.84	1.95	0.64	0.48	0.49	0.46	0.50	0.10	0.08	0.46
91	53.4	5.31	4.08	1.38	1.12	1.11	1.15	1.08	0.19	0.15	0.91
88	78.5	6.01	4.45	1.75	1.43	1.48	1.36	1.44	0.25	0.24	1.16
84	89.2	7.94	5.55	2.05	1.65	1.68	1.69	1.11	0.22	0.31	1.38

TABLE I—*Continued.*

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	Gl(OAc)$_3$	MeOBu	$PhCH_2OAc$	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
				Carcinoma residues after extraction.							
	mg.										
79	33.3	3.61	2.33	0.77	0.35	0.30	0.33	0.18	0.10	0.04	0.00
84	66.7	5.01	4.31	1.41	0.74	0.78	0.80	0.30	0.06	0.04	0.47
				Leg muscle extracts.							
95 B	8.9	0.38	0.18	0.17	0.13	0.08	0.05	0.22	0.00	0.08	0.00
95 A	65.7	2.16	1.15	1.74	1.20	0.98	0.72	1.83	0.33	0.67	0.76
84 RT	59.7	2.01	1.18	2.01	1.06	0.99	0.82	1.04	0.45	0.66	0.91
64 RT	96.4	2.81	1.81	2.77	1.54	1.43	1.11	1.81	0.95	1.06	(0.33)
				Leg muscle residue after extraction.							
88 RT	66.7	2.19	0.81	2.04	0.35	0.64	0.40	1.39	0.27	0.32	0.29
				Heart muscle extracts.							
65	11.9	1.00	0.57	1.10	0.36	0.44	0.28	0.27	0.14	0.31	0.00
63	15.2	1.38	0.76	2.01	0.55	0.58	0.40	0.68	0.38		
91 RT	6.1	0.97	0.36	0.92	0.13	0.28	0.12	0.62	0.12	0.22	0.07
64 RT	11.4	1.17	0.61	1.26	0.35	0.42	0.26	0.32	0.25	0.34	0.00
				Heart muscle residue after extraction.							
84 RT	66.7	1.71	1.04	1.55	0.22						
				Kidney extracts.							
95	8.9	8.64	5.56	3.64	3.06	4.88	2.53	6.09	0.57	0.92	
63	38.9	12.57	9.71	6.96	4.69	7.37	4.63	7.07	1.40		
91 RT	17.7	11.43	7.60	4.84	3.93	5.84	3.80	5.76	0.90	1.24	4.92
64 RT	29.7	13.71	9.14	5.26	4.30	6.26	3.15	4.91	1.11	1.62	4.31
				Kidney residues after extraction.							
81 RT	16.7	7.53	5.26	4.39	3.00	5.26	2.90	2.50	0.51	1.05	3.05
84 RT	26.7	9.15	7.14	4.94	3.98	5.91	4.11	3.16	0.91	0.86	4.29

TABLE I—*Continued.*

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	$Gl(OAc)_3$	MeOBu	$PhCH_2OAc$	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
					Lung extracts.						
	mg.										
95	8.9	3.56	1.82	5.29	1.10	2.05	1.43	(5.66)	0.72	1.16	0.79
100 RT	8.9	3.40	1.82	4.79	0.69	1.42	1.07	3.92	0.77	0.98	0.97
91 RT	15.1	4.39	2.40	6.23	1.19	2.41	1.77	4.41	1.46	1.32	1.47
88 RT	20.4	4.50	2.52	6.29	1.28	2.64	2.01	4.05	1.35	1.27	1.76
					Lung residues after extraction.						
88 RT	16.7	3.56	1.31	4.36	0.42	1.23	0.88	2.77	0.70	0.72	0.75
84 RT	33.3	4.47	2.28	5.80	0.91	2.26	1.93	2.41	0.96	0.88	1.26
					Spleen extracts.						
95	8.9	5.58	3.79	4.90	0.61	0.56	0.76	0.81	0.00	0.00	0.31
63	25.8	9.59	7.22	8.30	1.32	1.32	1.51	1.40	0.42		
100 RT	8.9	5.30	3.55	3.67	0.53	0.55	0.76	0.46	0.05	0.03	0.44
75 RT	16.6	7.64	5.28	5.06	0.79	0.97	1.04	0.66	0.09	0.04	0.13
					Spleen residues after extraction.						
79 RT	16.7	3.73	2.45	3.46	0.19	0.20	0.19	0.34	0.05	0.00	0.00
84 RT	33.3	5.81	4.20	7.47	0.47	0.56	0.75	1.07			
					Testes extracts.						
95	8.9	7.06	4.04	(2.92)	0.79	1.38	1.16	3.74	0.55	0.72	
65	35.3	11.30	8.10	7.17	1.93	4.55	3.21	4.82	1.49	1.67	1.64
91 RT	19.6	8.76	5.95	5.49	1.11	2.30	1.96	4.18	1.23	1.16	1.27
88 RT	26.5	8.72	6.16	7.20	1.65	3.20	2.75	5.20	1.41	1.36	1.78
					Testes residues after extraction.						
83 RT	16.7	4.32	1.88	4.96	0.47	1.42	0.76	2.20	0.63	1.21	(0.31)
84 RT	33.3	6.54	3.49	5.54	1.26	2.62	2.40	2.87	1.14	1.12	1.38

TABLE I—*Continued.*

Experiment No.	Tumor or tissue extracted per cc. solution tested.	PhOAc	$Gl(OAc)_3$	MeOBu	$PhCH_2OAc$	EtOAc	MeOAc	EtOBu	MeOBz	EtOBz	IsobuOAc
					Brain extracts.						
	mg.										
95	8.9	1.33	0.79	0.04	0.20	0.15	0.11	0.00	0.00	0.00	0.00
67	21.6	1.85	1.17	0.24	0.32	0.30	0.27	0.00	0.00	0.00	0.00
91 RT	23.2	2.62	1.86	0.39	0.53	0.44	0.45	0.19	0.00	0.02	0.37
88 RT	32.1	3.18	2.07	0.43	0.61	0.48	0.51	0.25	0.00	0.04	0.40
					Brain residue after extraction.						
85 RT	66.7	2.80	2.45	0.56	0.61	0.57	0.55	0.16	0.02		
					Liver extracts.						
95 B	8.9	7.66	4.40	4.87	2.10	2.58	1.61	4.46	0.61	0.82	
95 A	52.3	11.00	10.50	10.62	4.16	7.35	5.27	8.22	1.76	1.79	5.52
63	93.3	12.54	10.78	11.93	5.48	9.02	6.58	8.91	2.84	2.39	5.52
97 RT	8.9	9.39	6.21	7.15	4.14	4.30	2.66	7.04	0.85	1.19	4.41
91 RT	58.0	14.27	11.88	11.38	7.46	8.81	6.95	9.13	2.59	2.29	7.30
76 RT	87.7	14.09	11.64	10.45	4.50	6.98	5.40	6.81	2.29	1.91	4.69
					Liver residues after extraction.						
84 RT	33.3	10.48	8.96	9.67	6.67	8.43	7.41	6.13	1.61	1.76	5.98
88 RT	50.0	9.80	9.49	12.14	5.26	8.84	5.92	9.09	1.79	1.86	7.01

A number of charts will next be presented in which are shown the relative actions of the different tissues on the various esters, as percentages of the greatest action in that series. The absolute actions found are not involved directly in these charts although, of course, the relative actions are calculated from them.

The results for the rat tumor and different rat tissues will be discussed briefly in connection with the individual figures. The order in which the esters are plotted as abscissæ is entirely arbitrary. The same order is used in every plot throughout this chapter. The ordinates show amounts of hydrolysis in terms of percentage action upon equivalent amounts of the indicated esters which are plotted at equidistant intervals on the abscissa axis.

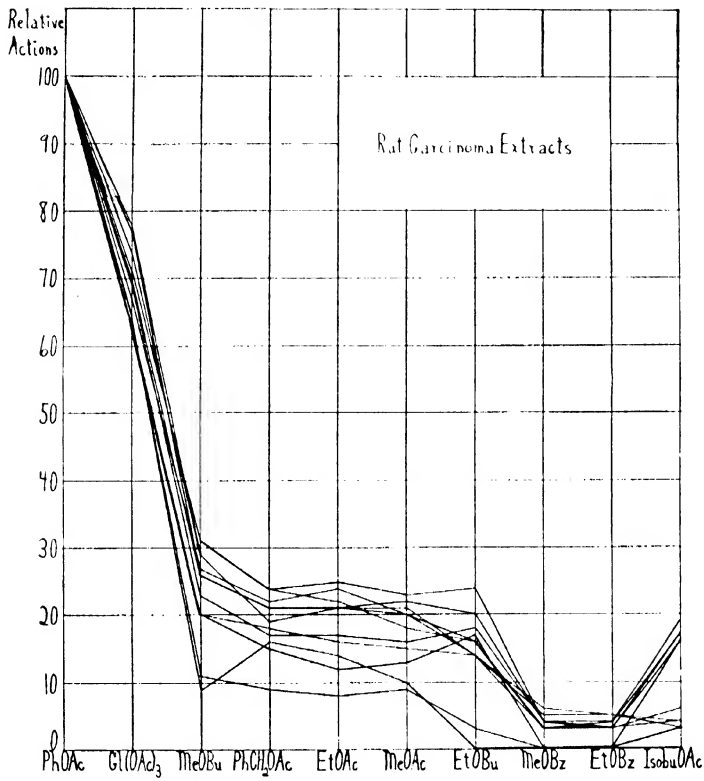


FIG. 1. Carcinoma extracts. Each curve represents the results of one series with extracts of tumors grown for 3 to 4 weeks in from six to twelve rats. The curves coincide quite closely when it is considered that in addition to the errors incidental to the various manipulations, very different amounts of tumor were extracted in the different series. There is evidently a definite relation between the actions on the various esters, not always clearly indicated when two esters only are considered, but shown definitely by the general nature of the complete curves. With isobutyl acetate and ethyl butyrate two sets of results are apparent. This is due to the fact that in the later experiments purer esters were used showing greater actions. As for a more detailed discussion it may be pointed out that with PhOAc taken as 100, $Gl(OAc)_3$ ranged from 63 to 78, and the other esters less than 30. No definite differences were observable between the corresponding methyl and ethyl esters, while $PhCH_2OAc$ was not much different from MeOAc and EtOAc.

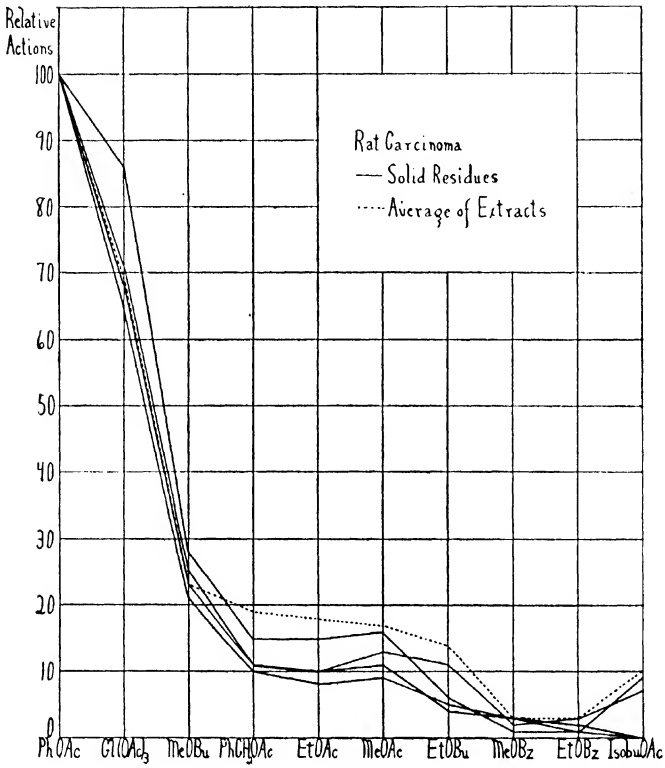


FIG. 2. Carcinoma. Solid residues. Since it is conceivable that the actions of extracts of tumors or tissues on esters may be different from those of the whole tumors or tissues, the solid residues after extraction were tested in a number of cases. The curves for the carcinoma residues show essentially the same pictures as the extracts. Because of the necessity of weighing the moist residues with the accompanying inaccuracy, the relative actions showed in some cases greater variations than did the extracts. In general, it may be said that for all the materials studied, the solid residues gave essentially the same types of relative actions as did the corresponding extracts. This eliminates the possibility that the relations observed are due mainly to solubility differences of the different ester-hydrolyzing enzymes.

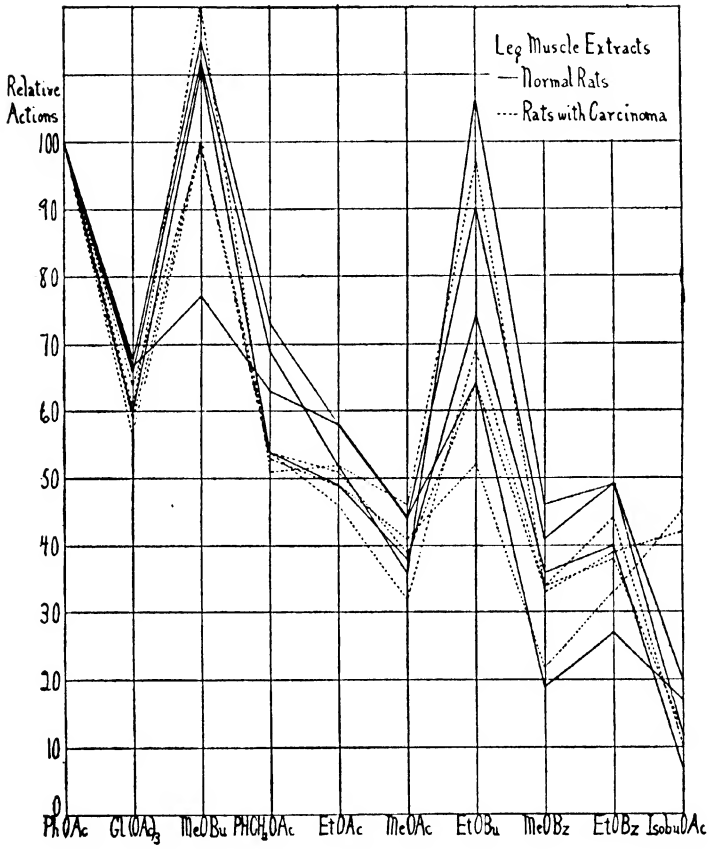


FIG. 3. Leg muscle extracts (normal and tumor-bearing rats). An entirely different picture from that of the tumor is shown by the leg muscle extracts. Some of the differences in the ethyl butyrate and isobutyl acetate results are due to the different samples of ester used. There are greater irregularities in the different curves of relative actions because of the smaller absolute actions and consequent greater influence of the experimental errors. The butyrates gave results as high as, or higher than, PhOAc. $PhCH_2OAc$ actions were larger than EtOAc and MeOAc (true for no other rat tissue studied). The presence of tumors in the rats exerted no influence on the actions.

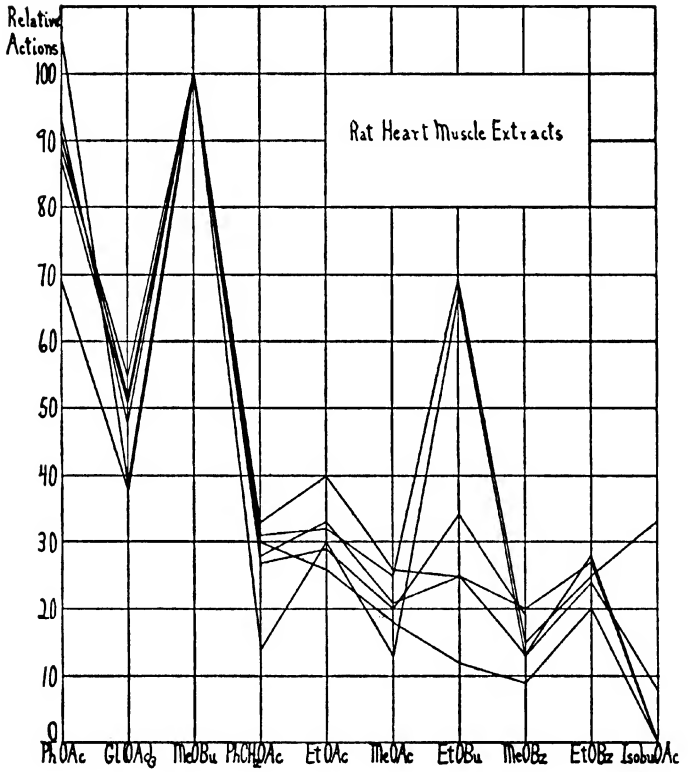


FIG. 4. Heart muscle extracts. The small absolute actions due to small amounts of material cause greater irregularities in the curves. The results differ from those of the leg muscle extracts in the smaller values for $Gl(OAc)_3$, $PhCH_2OAc$, EtOAc, and MeOAc.

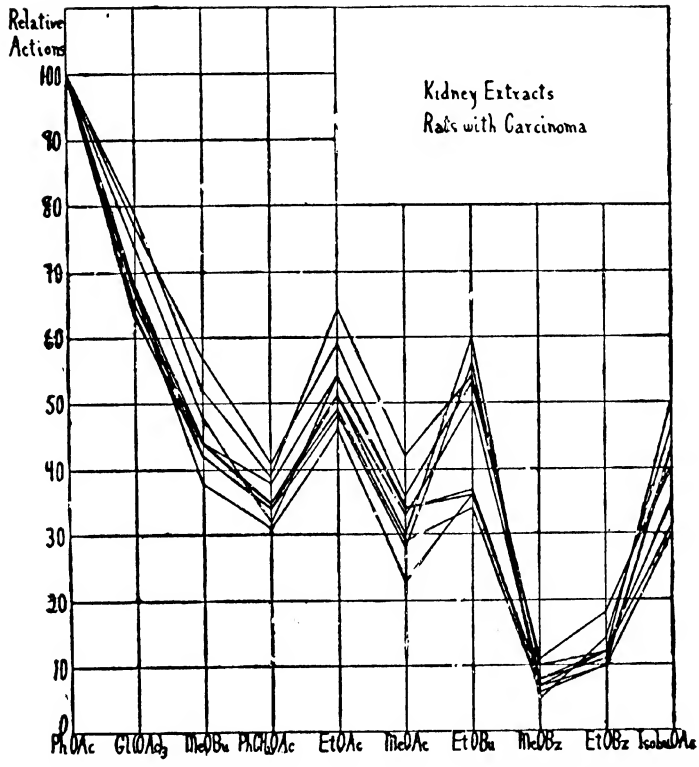


FIG. 5. Kidney extracts (tumor-bearing rats). Actions for ethyl esters as high as, or higher than, for methyl esters; while the acetates, aside from PhOAc, were as large as the corresponding butyrates.

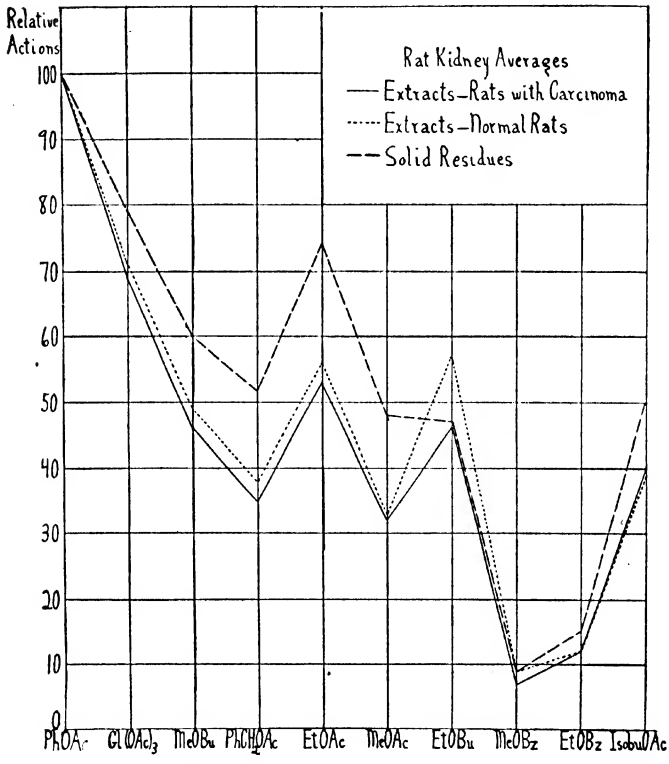


FIG. 6. Kidney averages (normal and tumor-bearing rats and solid residues). The averages show that the extracts of normal rat kidneys, of kidneys of tumor-bearing rats and of the residues after extraction gave essentially the same results. As usual, the results with the residues showed greater irregularities.

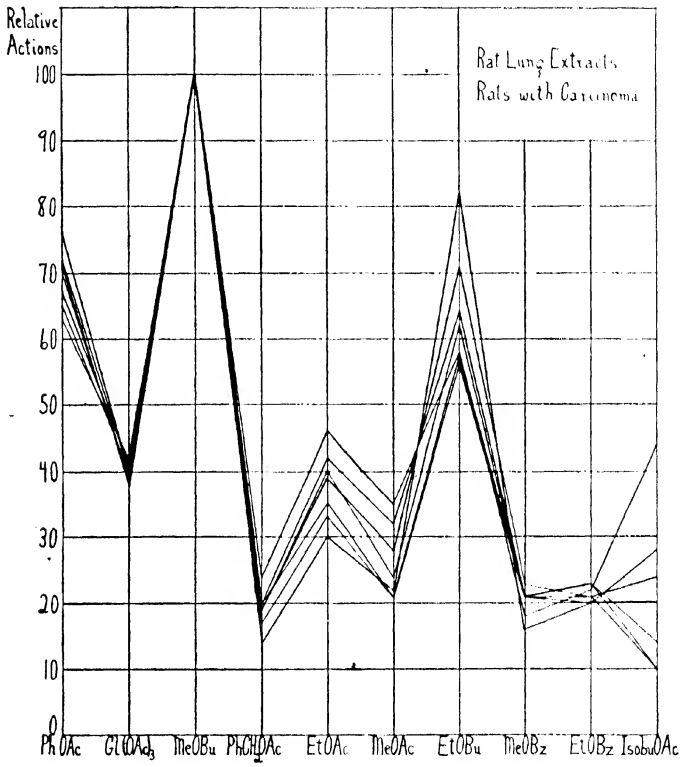


FIG. 7. Lung extracts (tumor-bearing rats). The large actions on MeOBu are striking. As for the rest, while the general picture is different, it is hardly necessary to call attention to the details.

FIG. 8. Lungs. Solid residues. The picture is very similar to that of the extracts, with the usual greater irregularities.

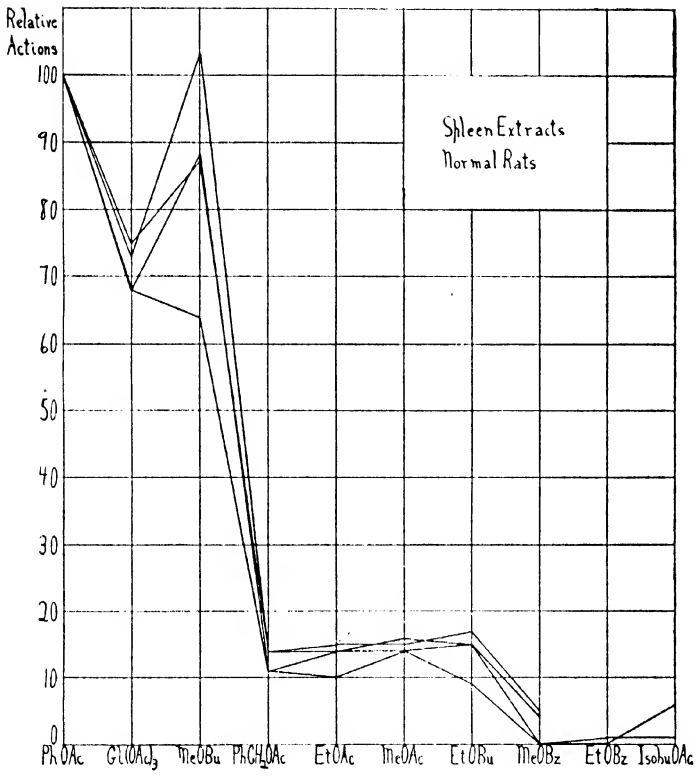


FIG. 9. Spleen extracts (normal rats).

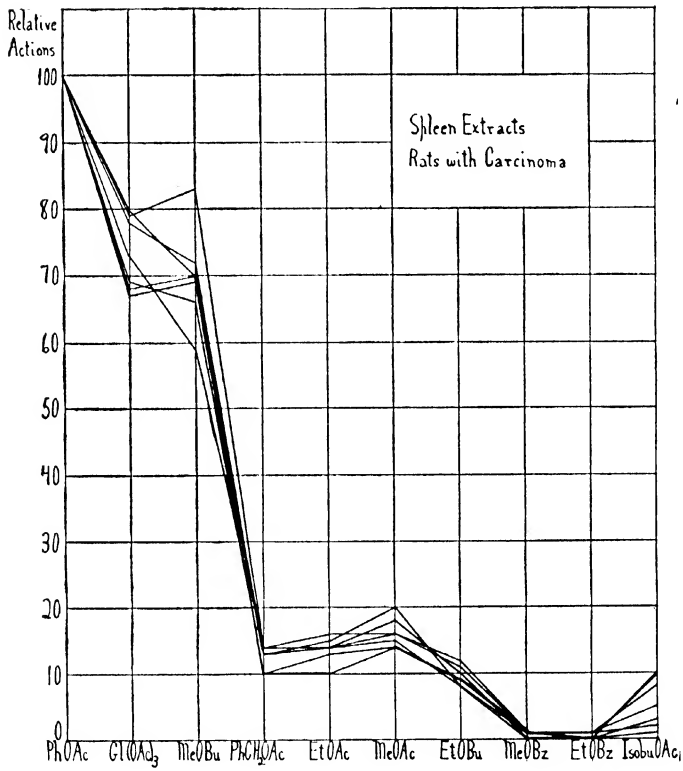


FIG. 10. Spleen extracts (tumor-bearing rats).

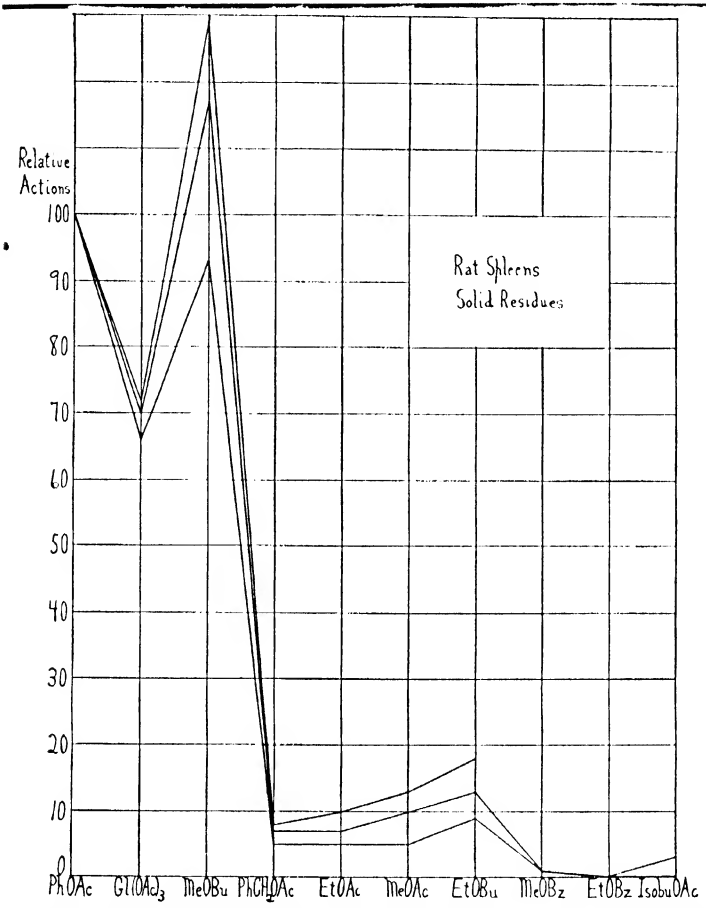


FIG. 11. Spleen. Solid residues. Figs. 9, 10, and 11 may be considered together. The pictures are much the same and similar to the tumor picture except for the methyl butyrate actions. These are somewhat irregular in all three charts. This may point to possible solubility differences for the different ester-hydrolyzing enzymes in the spleen. However, it is due more probably to a greater sensitiveness to external accidental influences of the methyl butyrate hydrolysis reaction. Similar influences, although much smaller in magnitude, were observed in some of the actions of other tissues. Although the methyl butyrate actions were somewhat irregular, still the action was in every case 60 or more (as compared with phenyl acetate 100) so that there is no possibility of confusion with the tumor picture. Also, although the actions for the remaining esters show relations similar to those with the tumor, still a minor difference is observable in that the spleen actions on the whole are smaller (in comparison with the first three esters)

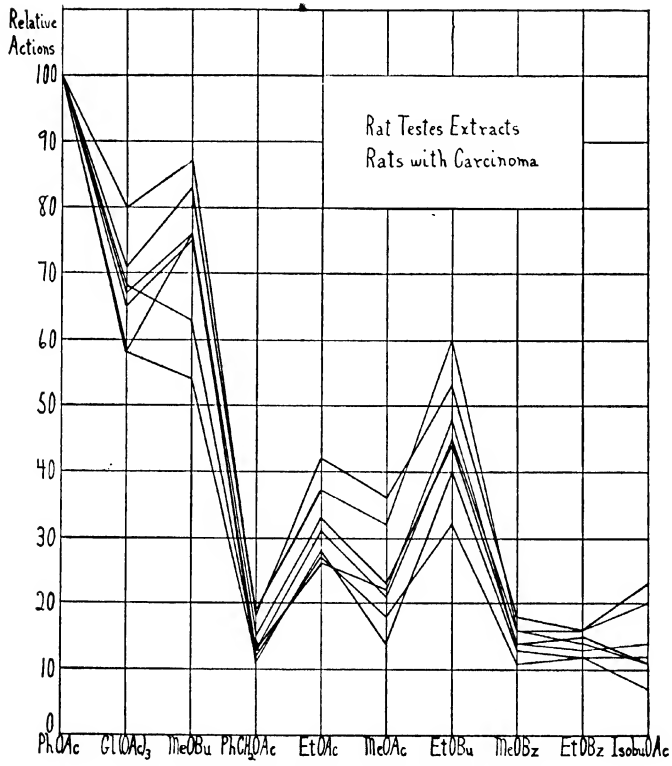


FIG. 12. Testes extracts (tumor-bearing rats).

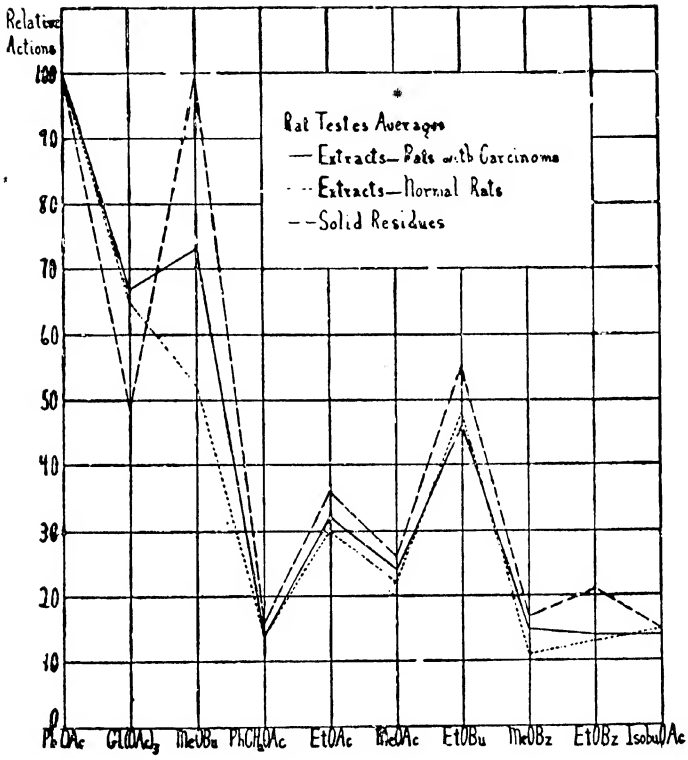


FIG. 13. Testes averages (normal and tumor-bearing rats and solid residues). The testes actions on methyl butyrate appear to be quite irregular, while those of glyceryl triacetate somewhat less so. However, if one of the other esters, not phenyl acetate, were used as the standard, much of the irregularity would disappear except for the phenyl acetate results. This is true especially for Fig. 12. The solid residues, as usual, showed greater irregularities than did the extracts. It may be noted that the butyrate actions were greater than the corresponding acetate actions. While the testes results are not as clean-cut as those of some of the other tissues, still, in comparing the different charts, the testes "picture" is fairly characteristic.

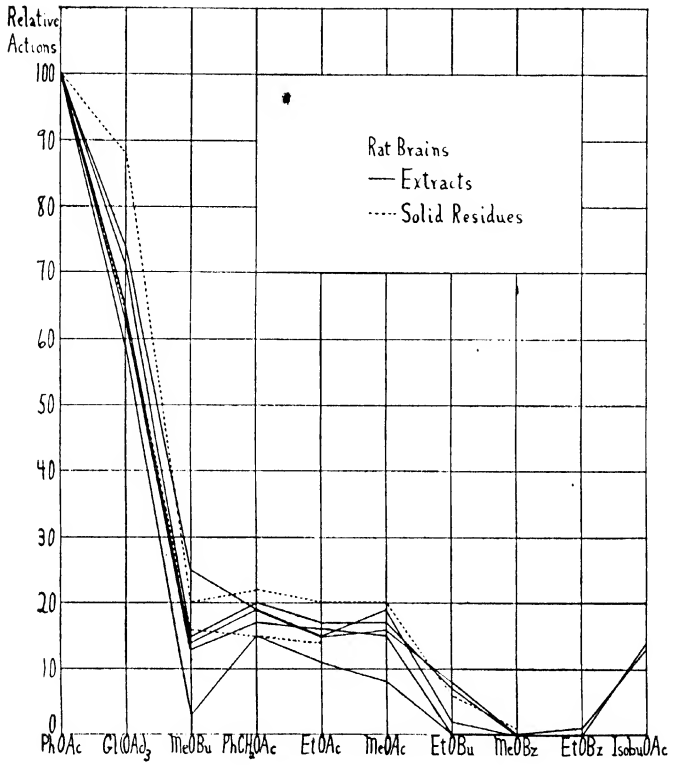


FIG. 14. Brain. Extracts and solid residues. These curves show greater similarities to the tumor curves than any other tissue. The absolute magnitudes of the actions as shown in Table I are not very different either, although somewhat smaller as a rule. The differences between the two pictures are of minor character and consist essentially in the smaller relative brain actions on the esters aside from methyl butyrate, and the fact that very little or no action was observed on ethyl butyrate and the benzoates. Also, it may be pointed out that with the isomeric esters, ethyl butyrate and isobutyl acetate, this is the only rat tissue in which the action was found to be markedly larger on the isobutyl acetate.

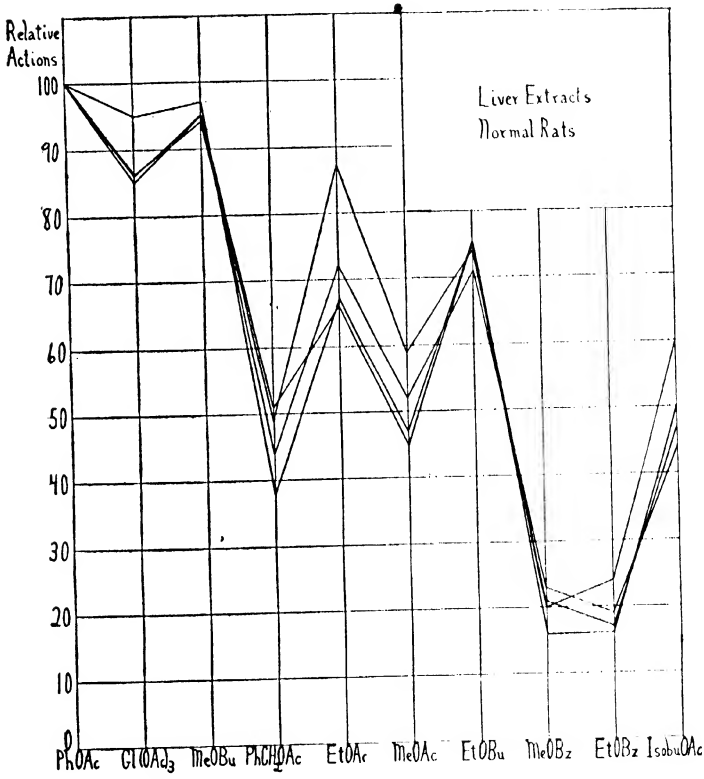


FIG. 15. Liver extracts (normal rats).

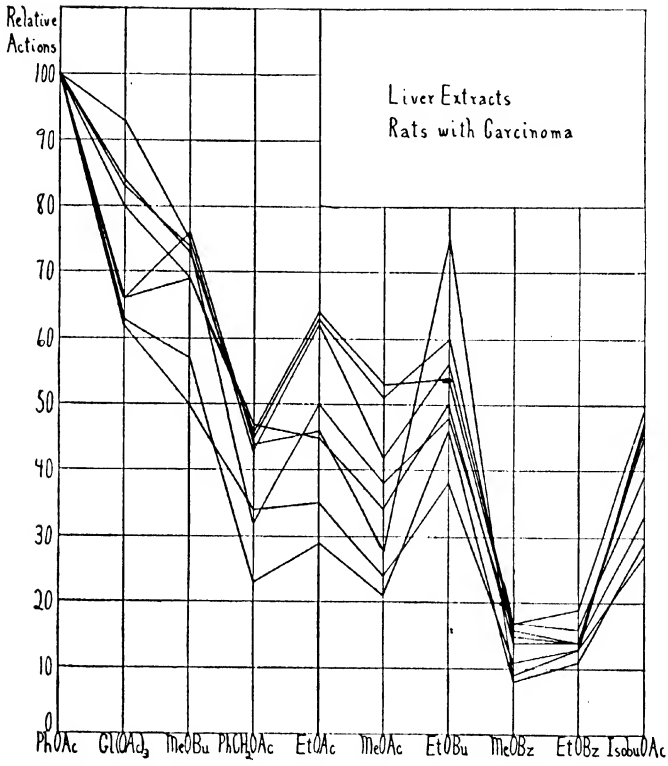


FIG. 16. Liver extracts (tumor-bearing rats). The curves for the extracts of normal rat livers (Fig. 15) show more general enzyme actions than do those with any of the other tissues. The results with the liver extracts of the tumor-bearing rats (Fig. 16) show distinctly different relations although there is an underlying similarity between the curves. In several of the experiments secondary tumors were found in the livers and removed as far as possible. It is probable that they were present in other cases, especially in the earlier experiments and not noticed since no microscopic examinations were made. The curves in FIG. 16, as a matter of fact, differ from those in Fig. 15 in that they begin to approach the characteristic tumor type (Fig. 1).

Emphasis has been placed so far more on the general pictures of the results than on any specific comparisons except in isolated cases. Some more specific relations will now be given.

Comparing the corresponding methyl and ethyl esters it is seen that for the butyrates the actions are greater in every case for the methyl ester than for the ethyl ester, except for the kidney where they are the same. For the acetates, the actions are less in every case for the methyl ester than for the ethyl ester except for the tumor, spleen, and brains, where they are the same. For the benzoates, the actions are less for the methyl ester with leg muscle, heart muscle, and kidney, and very nearly the same for the rest. The actions on methyl butyrate are greater in every case than on methyl acetate; greater for ethyl butyrate than for ethyl acetate with leg muscle, heart muscle, lungs, and testes; less with brains; and much the same with tumor, kidney, spleen, and liver.

In view of previous studies on glyceryl triacetate and ethyl butyrate,[5] a comparison of the former with the butyrates may be of interest. Comparing glyceryl triacetate and methyl butyrate, greater action was found for the latter with leg muscle, heart muscle, lung, spleen, and testes; comparing glyceryl triacetate and ethyl butyrate, greater action was found for the latter with leg muscle, heart muscle, and lung.

The isomeric esters might also be compared. For example, greater action was observed on phenyl acetate than on methyl benzoate in every case, but the ratio varied from 100:50 for leg muscle to 100:0 for spleen and brains. With benzyl acetate and ethyl benzoate, very nearly the same actions were observed with heart muscle, lungs, and testes, while benzyl acetate was higher with the rest. Isobutyl acetate gave larger actions than ethyl butyrate with brains; the reverse was true for the other materials.

It is obvious that the curves show characteristic relations or types for the tumor and each tissue. These relations as given hold only for the conditions of the experiments which were used, but it would naturally be possible to obtain analogous curves or types for other conditions. In addition to the general "pictures" a more detailed study of the results, taken in pairs of esters or groups of esters, brings out relations, a few of which were given, which make it

[5] K. G. Falk, *Jour. Amer. Chem. Soc. 36,* 1047 (1914); K. G. Falk and K. Sugiura, *Jour. Amer. Chem. Soc. 37,* 217 (1915); cf. also results given in Chapter VI.

possible to characterize definitely the action of a given tissue, or of a given extract as obtained by extraction of a tissue, perhaps unknown. In such comparisons, however, care must be taken not to assume definite relative actions for tissues and esters in cases where the absolute actions are small and introduce the possibility of error which might obscure the real relations. Reference to the results in Table I will show where the possibility of such errors might exist.

This raises the question of the absolute actions which were determined and which must necessarily be included for a proper understanding of the relations. It is advisable to compare extracts of the same amounts of original tumor or tissues. Even so, since different groups of rats were used, the absolute actions were found to differ considerably at times. It was only possible to obtain complete series for all the tissues in a few cases, and it might be misleading to present these results as conclusive evidence of such absolute actions. It is preferable to state some general relations for the present.

The first striking fact which may be mentioned is the small value of the carcinoma action on all the esters. The heart and leg muscle and the brain actions are of the same order of magnitude, but the actions of the other tissues are very much greater. While there are differences which depend upon the ester used, it may be said that in general the actions were largest with the kidney and liver (not very different for the two), that the testes followed closely, and then the spleen and the lungs. While this statement is a very rough approximation to the facts, it may be pointed out that the order may be different in certain cases. Thus, the kidney is low with methyl butyrate while the lung and testes are high, etc. Such facts, however, do not invalidate the general statement of the relations.

Some of the results obtained for the protease actions of the tumor and tissue extracts are given in Table II. Only those for which the concentration corresponded to 8.9 mg. of tissue extracted per cc. of solution tested are given.

Considerable differences in the actions are evident. For the peptone, the order of decreasing magnitude of the actions is kidney, lungs, liver, spleen, testes, tumor, brains, and leg muscle. For the casein, the order is kidney, spleen, lungs, testes, liver and tumor, brains, and leg muscle. The small absolute values of the carcinoma actions are striking again, as well as the high value of the kidney.

Table II.

Protease Actions (Formol Method) of the Rat Carcinoma and Tissue Extracts.

Experiment No.	Tumor or Tissue	Actions in tenths of milli-equivalents	
		Peptone	Casein
96	Carcinoma	0.65	0.28
97	"	0.77	0.17
95	Leg Muscle	0.26	0.13
95	Kidney	2.40	2.31
100 RT	"	2.39	2.27
95	Lung	1.32	0.68
100 RT	"	1.20	0.41
95	Spleen	0.92	0.74
100 RT	"	0.95	0.51
95	Testes	0.90	0.47
95	Brains	0.49	0.15
95	Liver	1.12	0.29
97 RT	"	1.27	0.37

Also, the carcinoma showed greater action than the leg muscle. The order of the actions is obviously different for the two protein preparations.

The relative actions on the two preparations cannot be compared as readily as with the ester-hydrolyzing actions, since only two substrates were used, and also because a number of the actions, especially on the casein, were too small to use without introducing considerable errors into the relative actions. Even so, it may be pointed out that the ratio of the two actions for the kidney was very nearly unity, and that the relative action on the peptone became greater in approximately the order spleen, testes, lung, leg muscle, tumor, brain, and liver. For the liver the ratio of the actions was nearly 4 to 1.

It is evident that with a number of different protein substrates, series of results with different tissues would be obtained, which, when compared in the same way as the ester-hydrolyzing actions, would show analogous results. The few results given here for the protease actions are so similar to the lipase actions which were given

in greater detail, that it is perhaps permissible to consider that these lipase results are of deeper significance as regards the general enzyme characters of the materials than might be expected at first sight.

The results which have been presented show definite characteristic "pictures" for the ester-hydrolyzing actions of the rat tumor and tissues. The protease actions were not studied in as great detail, but enough has been presented with these to show that analogous "pictures" might be developed with a number of protein preparations to make the same sort of differentiation possible. The relative actions of the various enzyme materials proved to be of the most significance in the interpretation of the results, but the absolute actions should also be considered. They aid in the study of the relative actions and also, in some cases, bring out interesting relations which might otherwise be ignored.

Several facts must be emphasized in the discussion of the results. In the first place, the curves presented and the relations developed refer to one special set of experimental conditions. While such curves and relations undoubtedly are typical of all studies of this nature, the details of the relations developed under different experimental conditions would unquestionably be quite different.

Secondly, the small absolute values for the enzyme actions of the tumor, in comparison with the enzyme actions of a number of tissues, are striking. Apparently, the magnitudes of the enzyme actions are not characteristic of these tumors or "abnormal" growths, but rather certain characteristic types of action.

The results obtained with the rat tumor and tissues have been presented in some detail, since they may be taken to underlie in principle as well as in fact all the work along these lines.

The data obtained with the tissues of other animals will not be presented in as extended a form. Only the averages will be given in the figures which are plotted in the same way, and some general statements of the absolute values of the actions will be added. Some results found with human tissues are included. These were obtained on materials from autopsies and are not as definite as the results with animal tissues, partly because of the character of the material and partly because of the limited number of results.

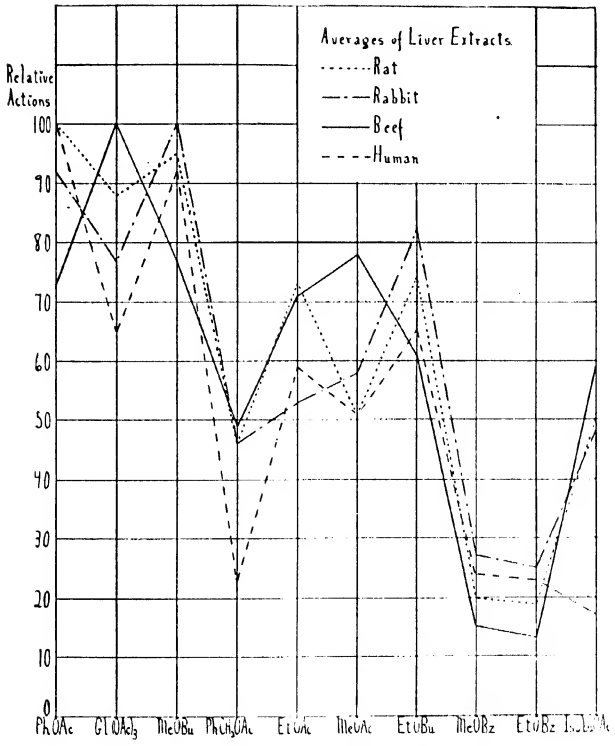


FIG. 17. Averages of Liver Extracts.

The rat, rabbit, and human livers show a general similarity; the beef liver being quite different. The low relative actions of the human liver on benzyl acetate and isobutyl acetate are to be noted, as well as the fact that the actions of all on phenyl acetate and methyl butyrate are of the same order of magnitude, and on ethyl butyrate not much less.

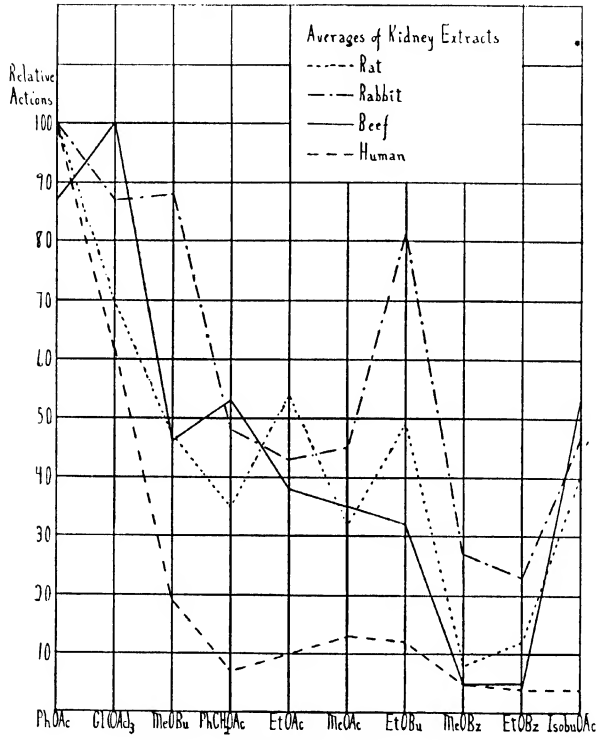


FIG. 18. Averages of Kidney Extracts.

The four curves show different pictures or types of actions. The relatively high actions on the butyrates shown by the rabbit extracts, the low actions on all esters except phenyl acetate and glyceryl triacetate by the human extracts, may be specially noted.

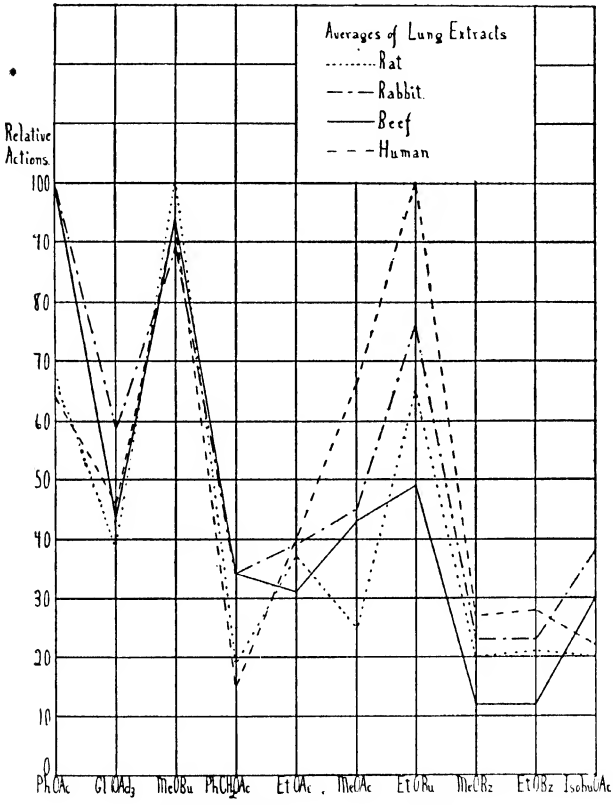


FIG. 19. Averages of Lung Extracts.

The various pictures are quite similar, although individual differences are apparent. The high values on methyl butyrate of all the extracts, and on ethyl butyrate, especially of the human extracts, may be noted. The differences in actions of the different extracts on various pairs and groups of the esters are sufficient to distinguish between them.

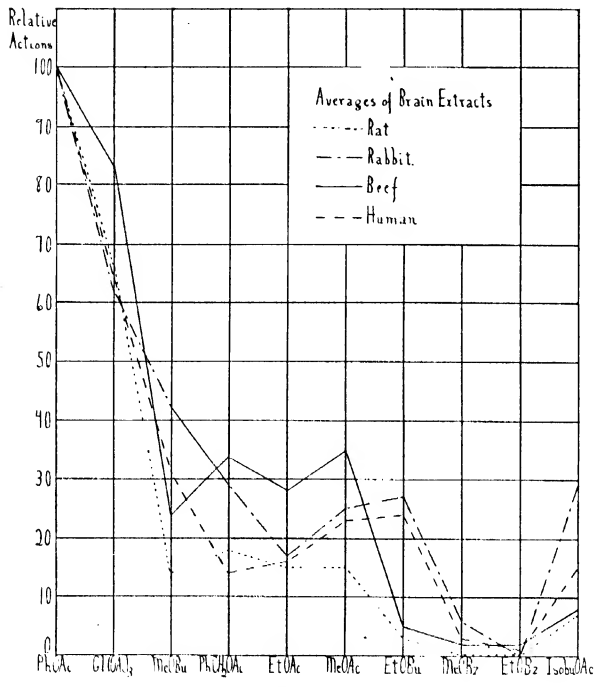


FIG. 20. Averages of Brain Extracts.

There is marked similarity in the actions of the different extracts. The differences which can be observed are of minor character and include the smaller relative actions of rat and beef extracts on ethyl butyrate, etc.

The absolute actions of the extracts of tissues from various sources will depend to a certain extent upon the ester used, since the "pictures" of the relative actions are different. Even so, some general conclusions may be stated. As a rule, the rat tissues are the most active, followed by rabbit, beef and human tissues in the order given. In some cases, the beef showed the greatest action (as with the liver for a number of esters), in others, the rabbit, but the general statement given may be accepted. The orders of the amounts of actions for the different tissues are 1. Liver, 2. Lung, 3. Kidney, 4. Brain, for the rabbit, beef and human, and 1. Kidney, 2. Liver, 3. Lung, 4. Brain, for the rat. The brain extracts showed the smallest absolute actions in every case, and the same picture of relative actions for every animal studied.

These results refer to the lipase or ester-hydrolyzing enzymes. The protease actions which were studied in a manner similar to the studies of rat tissues already given, did not lead to results as simple and as clear-cut as the lipase actions and therefore will not be given.

If general conclusions are permissible on the bases of these results on tissues from different animals, it may be stated that the corresponding tissues may show the same or different pictures for the relative enzyme actions and also for the values of the absolute actions. There appears to be no way of predicting the characters of the results. Some interesting relations, such as the similarity in the actions of the various brain extracts, are interesting and striking.

Some of the results which have been obtained with tumors of human origin, perhaps the most interesting part of this investigation, will now be given, in the forms of plots, with brief reports of the clinical and histological findings, and statements as to the absolute actions observed.

The absolute values of the actions of these tumors of human origin vary considerably among themselves, but, in general, for comparable concentrations, they are found to be small as compared with most tissues, either from human or other sources. They are more nearly of the order of magnitude of those found with muscle or brain extracts than the other tissues.

A number of results obtained with tumors which contained variable quantities of non-tumor tissue are not given. The pictures for these are irregular and do not appear to belong to any type, due unquestionably to the fact that mixtures were being studied.

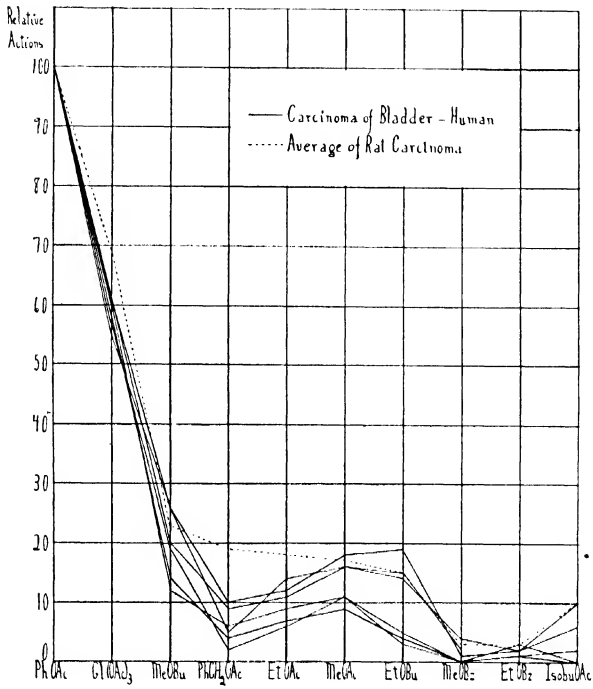


FIG. 21. Bladder Tumors of Human Origin.

The similarity of the various pictures is striking. The histological findings showed various types of carcinoma, etc., all of malignant character.

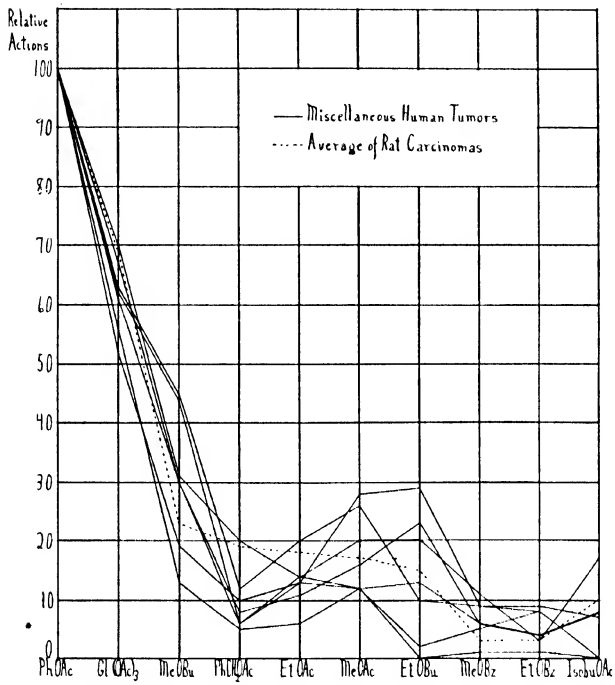


FIG. 22. Miscellaneous Tumors of Human Origin.

A number of pictures of results similar to the Flexner-Jobling rat carcinoma results, obtained with various tumors are shown. The histological findings were as follows: A. Metastasis in spleen, transitional type, carcinoma of either testis or spermatic cord. B. Neuro-fibroma of thigh. C. Multilocular cystadeno-carcinoma, papillary. D. Metastasis from carcinoma of left ovary. E. Solid alveolar carcinoma of breast. F. Metastasis to liver from carcinoma of rectum. G. Teratoid of testicle. Because of the similarity of the "pictures" of these actions, the different curves are not labeled. However, it may be stated that the order in which the histological findings are given is that found for the decreasing relative actions on ethyl butyrate.

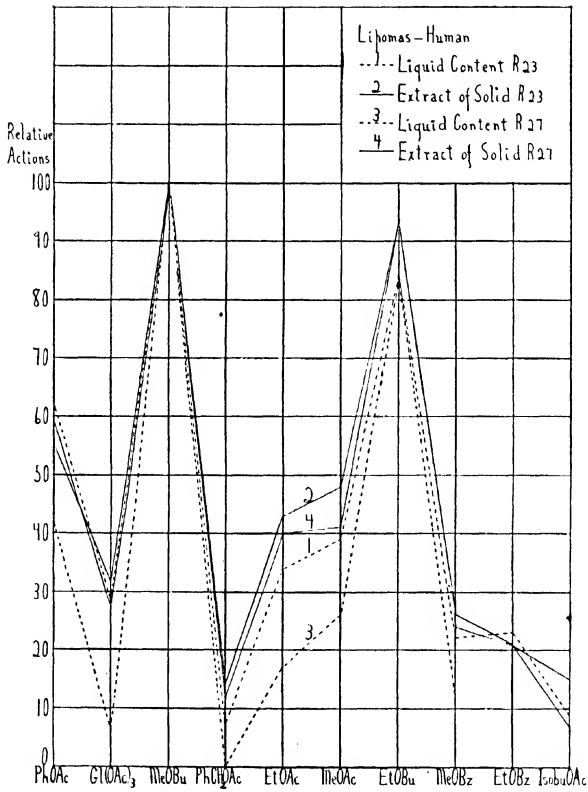


FIG. 23. Lipomas-Human.

The pictures found for these are entirely different from those given in the two preceding figures and the Flexner-Jobling rat carcinoma. It may be noted that these tumors are of fatty, benign character.

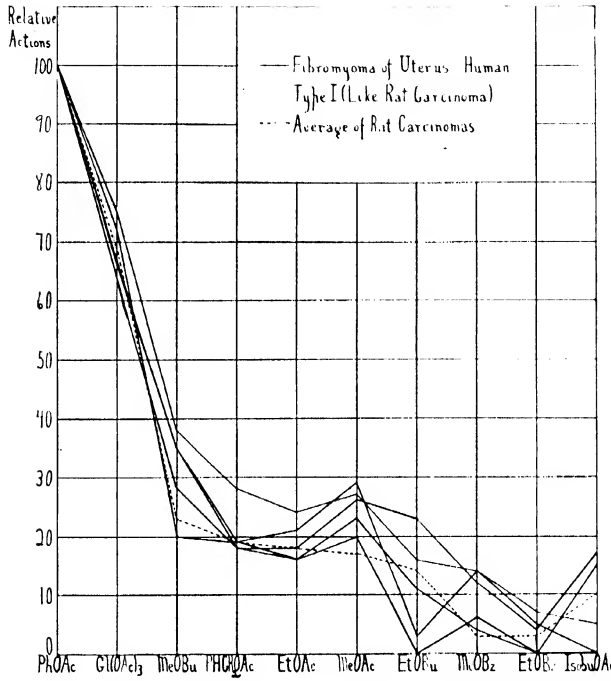


FIG. 24. Fibromyomas of Uterus. Type I. (Like Rat Carcinoma).

These curves show a definite likeness of a number of fibromyomas of the uterus to the rat carcinoma type. A number of different specimens gave the same picture as shown, each curve representing a surgical case. The histological findings were practically the same. It would serve no useful purpose here to give the individual histories.

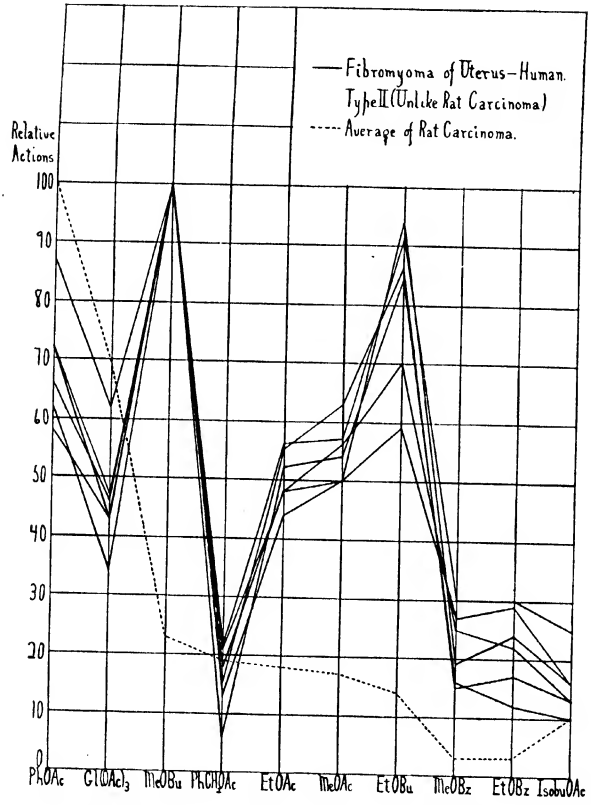


FIG. 25. Fibromyomas of Uterus. Type II. (Unlike Rat Carcinoma).

These curves are entirely different from those shown in Fig. 24, and different from the rat carcinoma, but show a very definite general type. For convenience, the different types shown in Figures 24 and 25 are designated Types I and II. The histological findings as given in the records were essentially the same as those given for the tumors shown in Fig. 24.

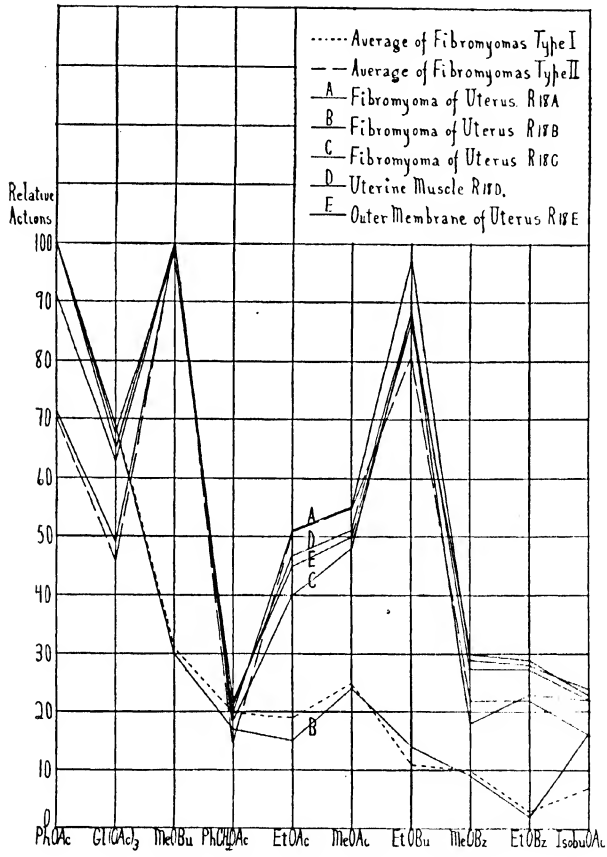


FIG. 26. Fibromyoma of Uterus (R18).

A. Hard, white mass, more or less spherical, about 4.5 inches in diameter.
B. Hard, white mass, 2.5 inches in diameter, slightly more color than A.
C. Small, hard, white masses, 0.5 to 1 inch in diameter, imbedded in uterine muscle.
D. Uterine muscle with outer membrane removed.
E. Outer membrane of uterus.

A, B, C, and E showed the same type of action (Type II); B, Type I, similar to the rat carcinoma. The Type II actions are evidently the same as the uterine muscle actions.

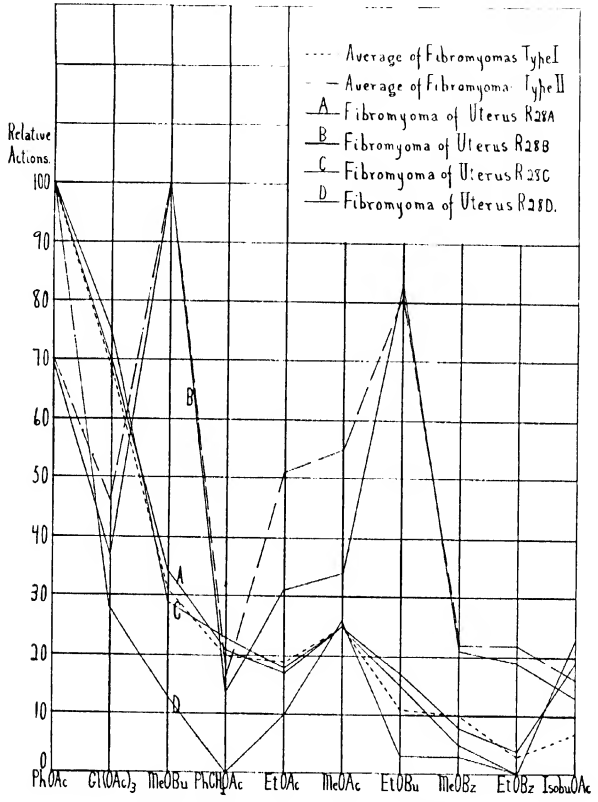


FIG. 27. Fibromyoma of Uterus (R28).

A. Hard, slightly colored masses; ¾ to 3 inches in diameter.
B. Two masses, 1.5 and 3 inches in diameter; red and slightly necrotic on border.
C. Two masses, 1.5 and 3 inches in diameter; in color between A and B.
D. Hard mass, 2 inches in diameter, with calcified scales on border.

Three of the masses of this specimen showed Type I actions similar to the rat carcinoma; one mass showed Type II actions, similar to uterine muscle.

The results on tumors of human origin which are shown in the last seven figures show a marked similarity in action for a number of different types of tumors. These actions are also similar to those shown by the Flexner-Jobling rat carcinoma. Although differences are shown in the histological findings in a number of these cases, these differences are not reflected in the relative enzyme actions as shown. It is true that more careful study and analysis of the results may show such differences of secondary nature, but for the purpose in view only the general relations will be given. There appears to be some underlying factor in tumor growth as shown by the enzyme actions, common to a large number and of fundamental significance. On the other hand, the results with the lipoma and Type II of the fibromyoma of the uterus show that all tumors do not fall into the same general grouping as regards the enzyme actions. This is especially true as regards the fibromyomas which in a number of cases showed definite differences in actions, even with different parts or masses from the same case, the one type of action being similar to that of the Flexner-Jobling rat carcinoma and various human tumors, the other type of action being similar to the uterine muscle actions. The figures show a few of these results; up to the present over 30 specimens of fibromyomas of the uterus have been studied in this way. As far as the records show, the histological findings of the fibroid tumors showing these two types of actions were essentially the same. No regular differences, either microscopic or macroscopic, were observed. The reasons for such differences in the enzyme actions of these tumors are therefore entirely unknown.

Despite the incomplete knowledge of the enzyme actions of certain tumor types, the regularities which have been found with the tumors of human origin point to definite similarities, and also differences, which indicate possibilities of further study along similar lines. It is hoped that it will be possible to publish shortly some results which will perhaps help to clear up some of the relations found and to show their connection with other processes involving growth.

One further point may be taken up in this chapter. It is possible that the presence of certain substances peculiar to a given tissue might modify an enzyme behavior common to all tissues. In this way the different pictures obtained for the various tissues would

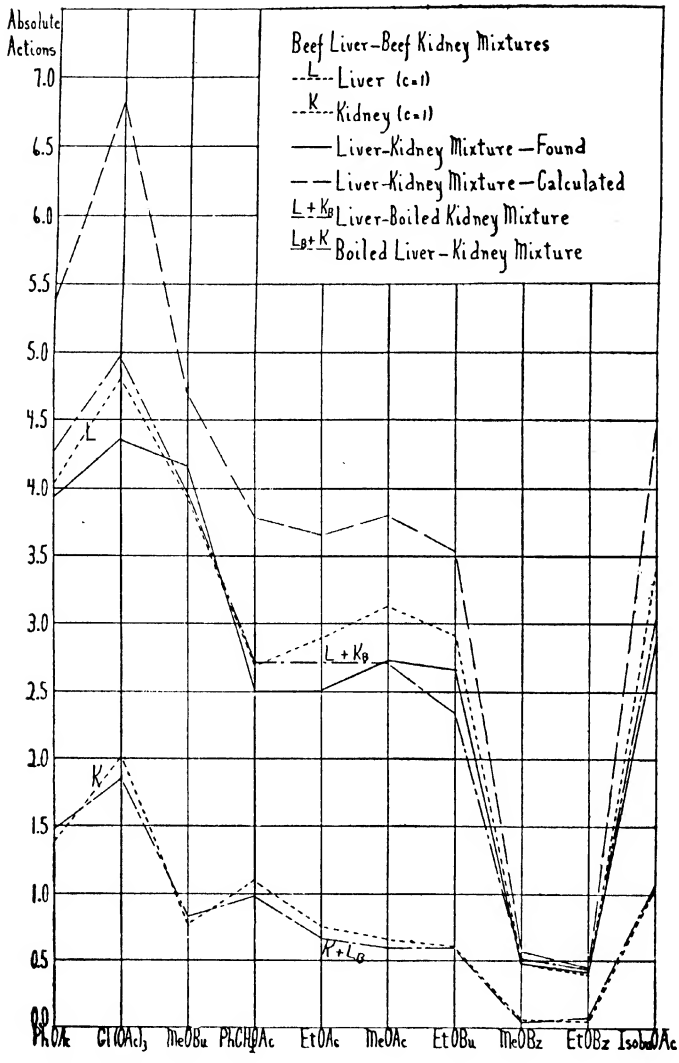


FIG. 28. In this and the following figures the ordinates show amounts of hydrolysis in tenths of milli-equivalents of acid formed from equivalent

amounts of the indicated esters which are plotted at equidistant intervals on the abscissa axis.

Beef liver-beef kidney mixtures. The kidney and boiled liver-kidney actions were practically identical, as were also the liver and boiled kidney-liver actions. The liver-kidney mixture was, if anything, slightly less active than the boiled kidney-liver mixture, and definitely less active than the sum of the liver and kidney actions (upper curve). The types of actions (relative actions on the various esters) were very nearly the same for the "Found" and "Calculated" mixtures. These would be more obvious if the relative actions on the percentage scale had been plotted in place of the absolute actions as such. The fact that the "Calculated" actions were greater than the "Found" actions may be taken only as evidence of the fact that the actions of even one tissue alone on the esters are not proportional directly to the concentration of that tissue in the extract. The application to the results with mixtures of two different tissues follows from this relation.

The results of the mixture of the liver and kidney, because of a similarity of their relative actions separately on the different esters, are not so striking as those shown in Fig. 29.

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be accounted for without the necessity of assuming a characteristic enzyme behavior for each separate tissue. This explanation, if correct, would change the study from that of different, though related, enzymes in different tissues to a study of the actions of various substances on one enzyme or one group of enzymes. The view can be readily tested by studying mixtures of tissue extracts and of tumor and tissue extracts.

The experimental methods were the same as those used in obtaining the results already presented. The only additions to be made include the facts that in the mixtures of tissue extracts and of tumor-tissue extracts, 5 cc. of each extract were used and 5 cc. of water added to make up the usual 15 cc., and in preparing the boiled extracts, these were boiled over a free flame for 5 minutes, made up to original volume, and then 5 cc. portions diluted to 15 cc. with water, or mixed with 5 cc. of unboiled extract, 5 cc. of water added and tested in the usual way.

The results on the ester-hydrolyzing actions are presented in Figs. 28 to 33. They cover mixtures of beef kidney and beef lung; beef kidney and beef liver; and rat tumor with rat spleen; rat lung, rat kidney, and rat liver.

The results are plotted as absolute actions (tenths of milli-equivalents of esters hydrolyzed). The concentrations of tissue and tumor extracted corresponded to 8.9 to 9.0 mg. of each per cc. of final solution tested except for the rat tumor-rat liver mixture where the tumor concentration was doubled.

That the differences between the values for the "Found" and "Calculated" mixtures are due to concentration effects as stated may be shown by means of the results of Experiment M15 in Table III.

Table III.

Ester-Hydrolyzing Actions of Beef Liver and Beef Kidney Extracts Alone and Mixed.

	Absolute actions found with tissue extracts				Calculated one-half the sum of 5L and 5 K.
	5 cc.L*	5 cc. K	5 cc. L+ 5 cc. K	2.5 cc. L+ 2.5 cc. K	
PhOAc	2.92	1.00	3.15	2.09	1.96
Gl $(OAc)_3$	3.65	1.76	3.74	2.53	2.71
MeOBu	2.80	0.21	2.90	2.09	1.51
$PhCH_2OAc$	1.87	0.91	2.11	1.35	1.39
EtOAc	1.98	0.66	1.88	1.31	1.32
MeOAc	2.01	0.61	2.07	1.32	1.31
EtOBu	2.26	0.34	2.11	1.34	1.30
MeOBz	0.44	0.04	0.52	0.27	0.24
EtOBz	0.38	0.00	0.47	0.25	0.19
IsobuOAc	1.99	0.84	2.37	1.58	1.42

* L represents liver; and K, kidney.

Each solution was made up to 15 cc. and tested in the same way. Columns 2 and 3 in the table give the results obtained with the liver and kidney separately at a definite concentration for each; Column 4, the results for the mixtures of these same concentrations, the values being considerably less than the sums of the actions in Columns 2 and 3 except for the benzoates; Column 5, the results for the mixtures with the concentrations half those in Columns 2 and 3 (or in Column 4); and Column 6, the results calculated from the sums of the actions in Columns 2 and 3 and divided by 2. The results in the last two columns are comparable. They show striking agreements between the found and calculated results except for one ester, methyl butyrate. In other words, at similar concentrations for the mixtures the absolute actions as well as the relative actions of the mixture of tissue extracts are made up additively of the actions of the separate tissue extracts.

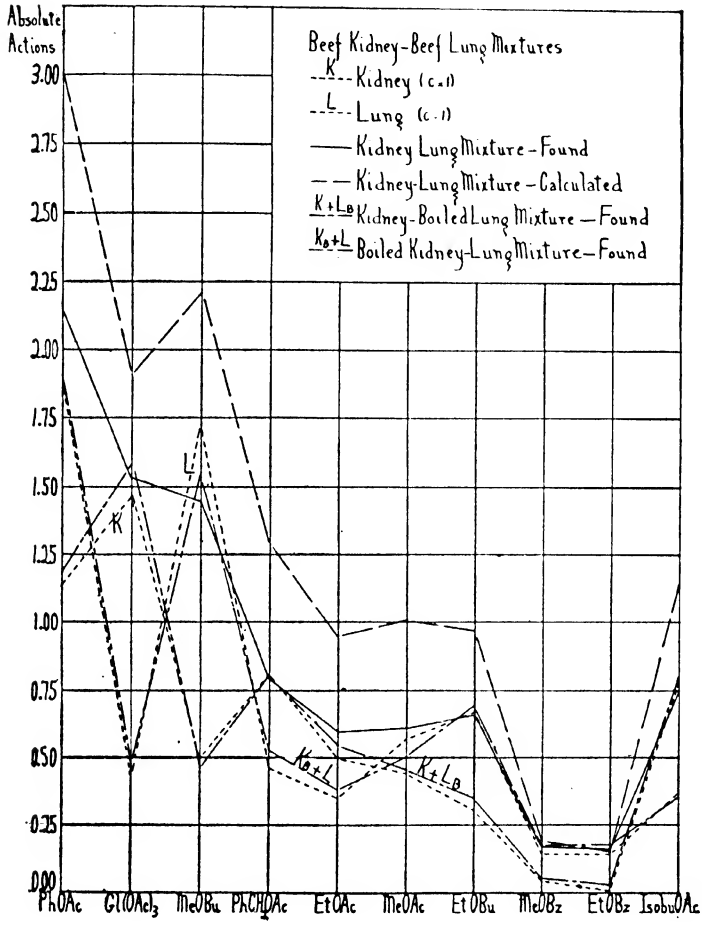

FIG. 29. Beef kidney-beef lung mixtures. The kidney and lung actions alone present entirely different pictures. Addition of boiled extracts did not affect the results in any way. The "Calculated" mixture gave absolute actions greater than the "Found" mixture, but the two pictures (relative actions or shapes of curves) are very nearly the same. The action of methyl butyrate (or perhaps glyceryl triacetate) only shows a small irregularity. If calculated in terms of percentages, the agreement between these relative actions would be still more apparent.

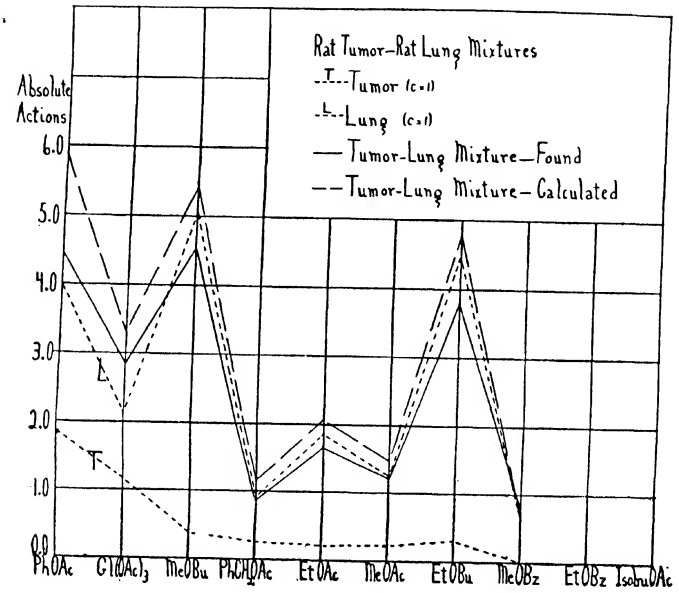

FIG. 30. Rat tumor-rat lung mixtures.

The results shown on Charts 30 to 33 may be considered together. They show clearly that the type or picture of the relative actions found in every mixture is essentially the same as that calculated from the separate actions. The absolute actions of the experimentally determined mixture are however again less in every case than the sums of the separate actions. This is not surprising, as already pointed out. It may be noted, on the other hand, first, that the absolute tumor actions in every case are much less than the absolute tissue actions; and second, the striking facts that the tumor-kidney mixtures show smaller absolute actions than the kidney alone. This is also true for some of the esters with the tumor-lung and tumor-spleen mixtures as compared with the lung and spleen actions alone. For the tumor-liver mixtures, the actions throughout are greater than the actions of the liver alone. The differences are not large in any case, and they are not such as to change the characters of the curves or pictures of the "Found" mixtures as compared with the "Calculated" mixtures. It is, of course, impossible to state at the present time whether these small effects are produced by the tissue acting on the tumor or *vice versa*. They can only be noted as being of secondary significance in connection with the main problem under investigation.

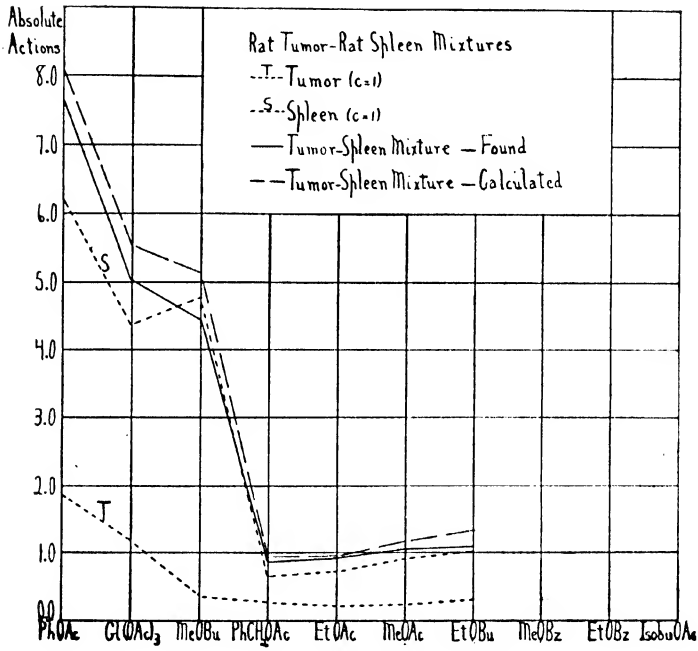

FIG. 31. Rat tumor-rat spleen mixtures.

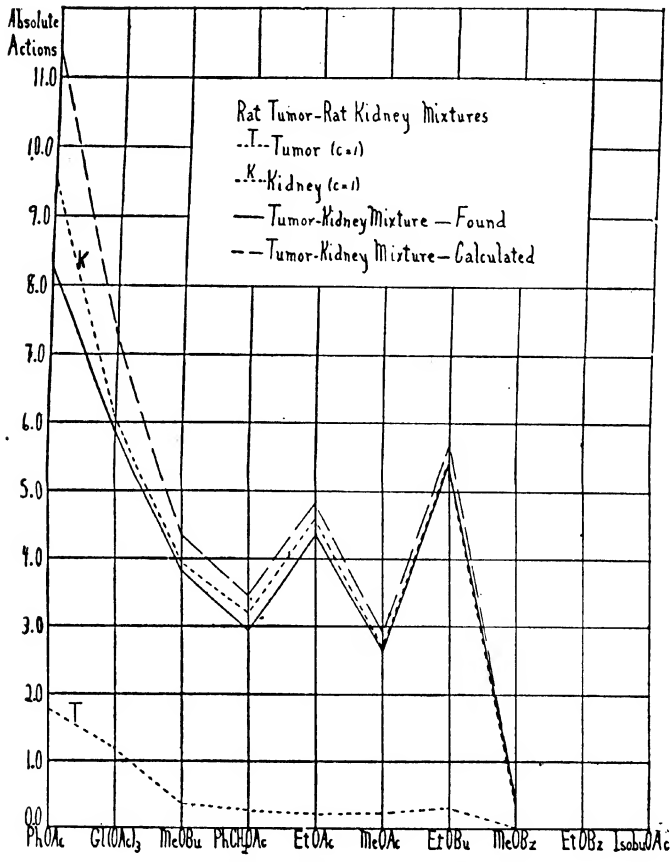

FIG. 32. Rat tumor-rat kidney mixtures.

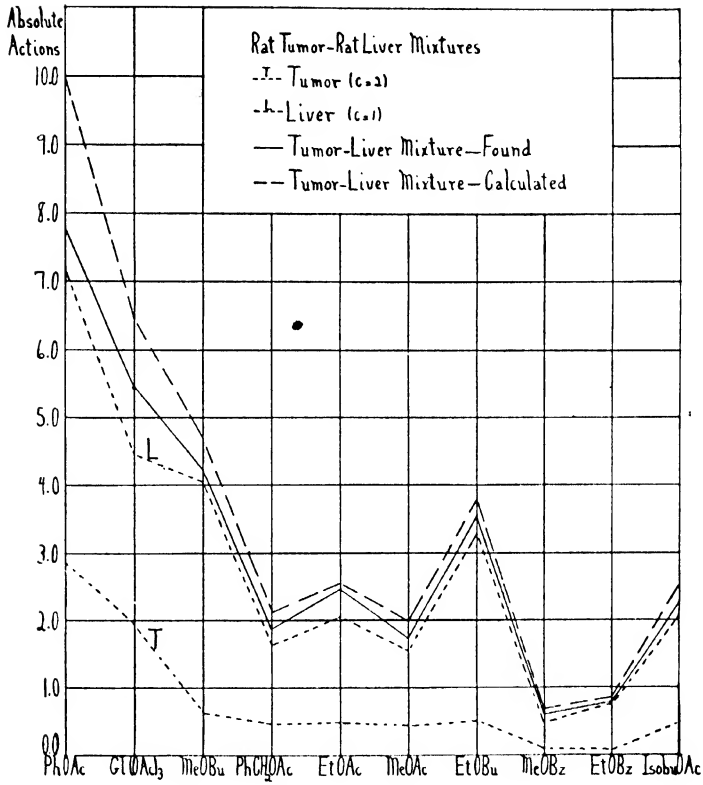

FIG. 33. Rat tumor-rat liver mixtures.

Some results with protease actions are shown in Table IV. The conditions of the experiments were the same as those stated earlier in this chapter. These results need little additional explanation. The ratios of the actions on the two preparations show very good agreements between the found and calculated (from the separate determinations) values in the first two experiments; not quite so good in the third, although in view of the magnitudes of the actions and the differences between the values of the ratios of the tumor and liver alone, no specific action of the one or the other can be said to have occurred. The absolute actions found for the mixtures are, in every case, less than the actions calculated, as with the ester-hydrolyzing actions, and may be referred to similar causes.

Table IV.

Protease Actions of Some Tissue and Tumor Extracts Alone and in Mixtures.

Experiment No.	Material tested	Actions on: Peptone	Actions on: Casein	Ratio of actions. Peptone: Casein
R 22 Beef	Kidney	1.69	0.48	3.52
	Liver	0.91	0.34	2.68
	Kidney + liver, found	1.93	0.60	3.22
	" + " calculated	2.60	0.82	3.17
R 31 Beef	Kidney	1.70	0.55	3.09
	Lung	0.76	0.35	2.17
	Kidney + lung, found	1.75	0.68	2.57
	" + " calculated	2.46	0.90	2.73
100 Rat	Tumor	0.94	0.39	2.41
	Liver	1.10	0.20	5.50
	Tumor + liver, found	1.30	0.47	2.77
	" + " calculated	2.04	0.59	3.46

These results show that, for the mixtures and actions studied, no specific influence on the enzyme action which can be ascribed to a substance or substances present in a given tissue or tumor peculiar to that material, was observed. The results given by Loevenhart[6] a number of years ago point in the same direction. He found that with extracts of the liver and pancreas of the dog and pig, the activity of mixtures for the lower fatty acid esters was

[6] A. S. Loevenhart, *J. Biol. Chem.* **2**, 427 (1906-07).

made up only of the activity of the ingredients. With olive oil and pancreas extracts in mixtures, increases over those calculated for the separate extracts were found. These, however, may well have been due to other causes such as, possibly, solubility effects, etc.

The evidence presented here relates in the main to the relative actions on the various substrates for each series. The absolute actions of mixtures were found in every case to be less than the sums of the actions of the constituents of the mixtures separately. As shown experimentally in one case, these differences may be referred mainly to the fact that the enzyme action of a given material in the case of those studied was not porportional to its concentration. Consequently it is not surprising that the action of a mixture containing two materials each of a definite concentration is less than the actions of the materials alone each of the same concentration as in the mixture. At the same time, certain minor influences of some of the tissue extracts on the absolute actions of the mixtures were observed which require confirmation and more extended study before anything definite can be said with reference to them.

The results which have been presented here at some length show some of the possible developments in the study of enzyme actions both from the point of view of the enzyme actions as such, and also in connection with the study of processes operating in living matter and the relations between such processes. Although the writer is directly interested in the latter phase of the problem, it may perhaps be permissible to say that the results so far obtained promise to systematize and possibly throw light upon a number of the reactions occurring in living matter and the relations between these. It may also be stated that some interesting data have been obtained in the study of the enzyme actions of embryonic tissues and their relations to the corresponding adult tissues and tumors, but it would lead too far to enter into these here.

Finally, in connection with the work which has been presented here, reference will be made to the papers which have already appeared.[7] The investigation is still in progress, and it is hoped that it will be possible to present additional communications in due time.

[7] K. G. Falk, H. M. Noyes, and K. Suglura, *J. Cancer Research 6*, 285 (1921); *J. Biol. Chem. 53*, 75 (1922); *55*, 653, *56*, 903 (1923); *59*, 183, 213, 224 (1924); *Jour. Amer. Chem. Soc. 46*, 1885 (1924).

X.—Present Status of the Enzyme Problem

In discussing the present status of the enzyme problem, the views presented will be those of a chemist who has worked on certain phases of the question, and whose personal outlook, inclinations, associations, and opportunities for study, will color his opinions to a certain extent. Most stress will therefore be placed on the chemical features of the problem. The views of a physiologist, of a botanist, or of a biologist, in discussing the same questions might well lead to entirely new viewpoints and emphasize different aspects of the problem. Bearing in mind the limitations indicated, a brief summary of some of the questions will be presented.

In the first place, it may be pointed out that no attempt has been made to describe and summarize all the enzyme work which has been recorded. A very complete and critical review of this nature is being published by H. von Euler.[1] It seems hardly necessary or advisable to attempt to duplicate such a summary. It has been the aim of the writer to bring out relationships of enzyme actions to other phenomena in chemistry, to present only data which would make such relationships clear, and to give some of the more recent work on enzyme actions in order to show the trend the study of the problems is taking.

A review of the topics treated in the earlier portions of this book may make the point of view clearer. In the first chapter the general problem of enzymes and enzyme actions was outlined and the general lines of investigation stated which appear at the present time to offer the most promise. These include such problems as the kinetics of enzyme actions, the comparative actions of various enzymes on different substrates, the purification of enzyme materials, and the study of enzymes as substances possessing definite chemical structures or configurations. The purpose of this monograph is to point out the progress which has been made in these lines of investigation. This method of treatment implies that

[1] Two parts have appeared at the time of writing: "Allgemeine Chemie der Enzyme," II Edition, Parts I and II, Munich and Wiesbaden, 1920 and 1922.

enzymes are to be considered as chemical substances which obey the laws of chemistry, and whose actions are explainable by the theories in vogue. In short, enzymes and enzyme actions form an integral and active part of chemical science, falling in line with present theories, suggesting new hypotheses, and together with other topics and fields of chemistry, correlating facts and relations.

In order to bring out these views, some of the general relations and theories of chemistry which are most closely related, and which apparently may be applied to enzyme actions more or less directly, are outlined in the first two chapters. Chapter I also includes some of the more recent views of chemical structure. In Chapter II are stated the elementary equations of chemical kinetics, since enzymes manifest their actions by changes in velocities of chemical reactions, with special emphasis placed on the limitations of the deductions, and a general theory of chemical reactions, including catalytic reactions is given as a necessary foundation for the further developments and classifications. The theoretical considerations given in these two chapters at first sight are not needed in the usual study of enzyme actions, but if the latter are to be treated as, and made part of, one of the general problems of chemistry, they must be included in some form. Generally, enzyme actions are spoken of as chemical actions and the subject disposed of in this way without stating what may be meant by this all-inclusive generalization. It appears to be advisable for further progress to be more specific even at the risk of finding the results which may be useful somewhat meagre in quantity, and the theoretical developments which have been given not always sound. Following the lines of reasoning indicated, in the third chapter some of the properties, relations, and theories involved in some of the simpler chemical reactions of use in enzyme studies are outlined. It is shown that, while much has been done in the way of systematizing the relations and many questionable points have been cleared up, a completely satisfactory explanation and theory of even the simplest and most carefully studied of these reactions is not at hand.

The more direct description of enzymes and enzymes actions is then taken up. Physical properties common to enzyme preparations are described in Chapter IV and chemical properties common to enzyme preparations in Chapter V. The distinction between physical and chemical properties is arbitrary and artificial to some extent

and is made mainly as a matter of convenience. The views on adsorption which have been suggested by others as possibly the main feature of enzyme actions are given. The relation between chemical structures and adsorption are discussed and the view upheld that the chemical structure is the predominating influence in these actions. The main chemical property considered is that connected with the acidity of the medium or mixture in which the enzyme action takes place. A number of questions connected with this property are taken up. Interesting parallelisms between the behaviors of indicators and of enzymes are pointed out. The fact that the hydrogen ion concentration affects most chemical reactions and that maximum or minimum influences on velocities may frequently be observed was also indicated. The specificities of enzyme actions are also considered briefly. The probable chemical structures of some enzymes and the purest and most satisfactory enzyme preparations which have been obtained are described in Chapter VI. These results indicate also the manner in which new enzyme problems may be taken up in so far as obtaining the necessary material is concerned. The mechanism of enzyme actions, based fundamentally on the kinetic equations given in Chapter II, is discussed in Chapter VII. The complicated nature of the reactions, the underlying principles involved, and some of the attempts at solving the relations are described. A careful study of some of the work which is outlined in this chapter taken in connection with the contents of Chapters II and III will serve to show the difficulties which are encountered in applying exact kinetic equations to the experimental work with enzymes and the necessity for making clear the various assumptions which are introduced in such measurements and calculations. A few of the industrial and laboratory uses and applications of enzymes are outlined in Chapter VIII. In Chapter IX recent work on some of the enzyme actions of tissues and tumors from various animal and human sources is given for the purpose of indicating a possible application of enzyme studies to a related field of science.

The enzyme studies of tissue and tumor materials described showed definite differences and similarities which may ultimately aid in throwing light on the problems involved in normal and (so-called) abnormal growth. The conditions of working under which these results were obtained were purposely kept as simple as possible,

no substance being added which might interfere with or modify the enzyme actions. Recently, Willstätter and Memmen[2] published some work along somewhat similar lines, in which the lipase actions of certain tissues were studied after the addition of substances such as albumin, sodium oleate, and calcium chloride, in order to retain the same conditions of testing in all the experiments. Unquestionably, these results of Willstätter and Memmen will prove to be of great interest and value, but from the point of view of the investigation described in Chapter IX the addition of such foreign substances would tend to complicate the conditions and probably obscure the very relations and comparative actions sought.

The colloidal property has been assumed in the past to be to a certain extent characteristic of enzymes. In the present treatment, the colloidal property as such has been ignored or at least relegated to a subordinate position in considering the actions. The enzyme substances are assumed to behave on chemical treatment just as other substances behave. The exact state of a substance, whether gaseous, liquid, solid, or in solution, will naturally modify the relations observed, but fundamentally the chemical reactions of a substance are based upon its chemical properties. There appears to be no valid reason to separate colloidal substances and to attempt to develop a new point of view in order to discuss the chemical reactions of such substances. Obviously, with a different set of substances, or with the same set of substances in a different state, experimental work may bring out some heretofore unknown or ignored property. The view is gaining ground rapidly that the attempts to develop a colloid chemistry distinct from the ordinary chemistry have failed and that the most satisfactory point of view lies in considering colloid chemistry as a part of chemistry, at the same time including those portions of physics which apply more directly to the phenomena. As stated already, the colloidal property of most enzyme preparations may be due to the fact that these are obtained from biological material. The enzyme property, however, is not necessarily connected with the colloidal property, although the stability of the enzyme property may, in a measure, be connected with the complexity of the molecule or micelle as a whole. It should therefore be possible to obtain the former even from biological material, in a crystalloidal or readily dialyzable

[2] R. Willstätter and F. Memmen, *Z. physiol. Chem.* *133*, 229 (1924).

form. The work of Willstätter on the purification of enzyme materials indicates some of the possibilities along such lines. This view, as well as other views to be outlined presently, may perhaps be thought to be too simple for the complex conditions which are met with in enzyme work. There is no intention of ignoring the great experimental difficulties which are encountered in enzyme work, and the very unsatisfactory material (chemically speaking) which must so frequently be handled. On the other hand, it is desired to throw out as far as possible complex theories of actions and to go back to definite chemical principles. In going back to such principles and relations, it is not meant that the phenomena can be treated at present as simply and as satisfactorily either from the experimental or the theoretical side as those phenomena which have been under investigation for hundreds of years. It is not known whether the presentation of such a point of view is successful in the present instance. Whether it is or not, it is the belief of the writer that this is the direction in which progress can be achieved.

The study of the chemical structures of enzymes is part of the study of the chemical structures of substances of biochemical origin. In isolating such substances from living matter, changes are frequently brought about by the reagents or treatments employed. Especially with substances of large molecular weight, colloidal substances, and those possessing optical activity, would changes be expected, which, while not detectable by many of the usual methods, still cause the substances to have different properties from those they possess *in vivo*. Much is known of the structures of simple substances obtained from biological material, but for the complex bodies, such as the proteins and starches, only the component parts and some of the methods of linking between these have been elucidated. It might appear therefore that the determination of enzyme structure would have to wait for the solution of the problem of the structure of other similar complex bodies. This, however, does not follow necessarily, and, in fact, it appears as if the problem might be reversed in some cases. The study of the structure of an enzyme is simplified by the fact that it is possible to follow a change in its structure by the change in activity. This, in a sense, furnishes an additional reagent and makes possible the study of an enzyme in a state more nearly approaching that in which it exists in the living

organism. There is therefore a possibility that the determination of the structures of enzymes will precede the determination of the structures of other biological materials.

Following this line further, an enzyme may be conceived of as consisting of a molecule showing specific properties, or of a definite grouping in a more complex molecule. This point of view was developed in some detail in Chapter VI, where it was considered that an enzyme grouping was responsible for a given action. In this connection, it is of interest to quote from a paper by Taylor published some years ago [3]: "Upon the basis of the current conception of fermentative acceleration as consisting in the establishment of intermediary reactions, the chemical properties of the different groups within the molecule would determine whether a certain substance could be an accelerator for a certain reaction; and a single molecule could in all correctness be assumed to contain different groups that would qualify it to act as accelerator for different actions . . . there is no reason why all the enzymic activities of the pancreatic juice (lipolytic, proteolytic, amylytic, inversion and coagulation) should not be ascribed to the different groups of one organic molecule." The possibilities of isomerism, structural, tautomeric, and stereochemical, are so numerous in a protein molecule, for example, as the latter is conceived of at the present time, that a great number of structures differing only in these ways, could be given, and presumably, given sufficient time and skill, be prepared in the laboratory.

For a number of years the writer has adopted the view that enzyme actions are due to certain definite groupings in a more or less complex molecule, that a definite enzyme grouping could be modified in its action by the presence of various atoms or groupings, distinct from the enzyme grouping, in the molecule, and that the complexity of the molecule as a whole played an important part, colloidal or otherwise, in retarding the transformation of the active grouping into an inactive one. Essentially similar views have been advocated by Michaelis, Euler, Willstätter, and a number of others, some having proposed such a conception of enzyme action years before the present writer, others later. The feature which it is desired to emphasize in this connection is not a question of deciding priority in these views, since it may be taken for granted that each worker came

[3] A. E. Taylor, *J. Biol. Chem.* 5, 400 (1909).

to the same general conclusion as a result of his own experimental work. The fact that there is considerable agreement, not complete it must be admitted, among the different workers with reference to the fundamental nature of enzyme actions is encouraging in that it gives a more satisfactory working basis, although it may be true that the general view presented is actually only the interpretation and working hypothesis natural for a chemist to suggest.

The question of specificity of enzyme actions may be taken up next. These specificities, as stated in an earlier chapter, are striking in many cases, but not unique considered as chemical phenomena. The most obvious reactions in which specificities are used are those included in Qualitative Chemical Analysis (and also Quantitative Analysis). In the reactions involving the identification of the metallic elements, these may be compared to the substrates in enzyme actions, and the reagents used to the enzyme preparations or materials. There are, in both cases, group reagents and individual reagents. With enzymes, for example, amylase, different proteases, emulsin, lipase, etc., act upon certain groups of substrates. Within each group there will be smaller differences for each individual substrate with the group reagent. The conditions must also be kept within certain limits. In qualitative analysis, similarly, hydrogen sulfide might be used as an example of a reagent showing group reactions with certain metallic elements in solution, as well as differences with the individuals in the group, while the conditions of the reaction (such as acidity or alkalinity, etc.) must be kept within certain limits. These analogies might be multiplied indefinitely. One set of phenomena is as remarkable as the other, but familiarity with the one has made these reactions commonplace, while the practical necessity for replacing definite chemical substances by substances as yet not as well characterized and therefore known by less familiar names, has resulted in enzyme actions and their specificities acquiring a certain air of mystery. This is unjustified, and their reactions are no more mysterious than are other chemical reactions.

In the same way, the specificities of the oxidizing enzymes may be compared to the actions of the different reagents used in the oxidation of inorganic and organic elements and compounds. The amount of space devoted to these enzymes in this monograph has been small compared with the space devoted to the enzymes which

influence hydrolysis reactions. Fundamentally, this is due to the unsystematic and merely qualitative knowledge of oxidation reactions of almost all organic compounds. The systematization of such reactions on some definite basis appears to be possible at present. The further developments in the study of the oxidizing enzymes will then be based upon these oxidizing relations and a satisfactory and reasonable choice of substrate will be possible, instead, as at present, of having the choice depend with each enzyme upon the personal preference of the experimeter.

The lock-and-key suggestion of Fischer[4] with regard to specificities of enzyme actions may also be taken up briefly. This view was developed from the actions of yeasts and other organisms. He extended it to the enzymes, sucrase and emulsin, acting upon the glucosides, and explained the actions by the assumption that the approach of the molecules which is necessary for the occurrence of the chemical process can take place only with similar geometrical structures of the reacting components (enzyme and substrate). To use a picture or model to illustrate the hypothesis, he suggested that enzyme and glucoside must fit in with one another as lock and key in order for them to exert a chemical action upon each other.

This view and analogy has always aroused great interest. It is a question, however, as to how far the analogy should be taken to hold in connection with elucidating the chemical structures of enzyme molecules. The basic idea of Fischer involves the view of an intermediate or primary addition compound between substrate and enzyme, a view for which various other lines of evidence have been developed. However, the use of the lock-and-key simile as more than an analogy appears to be open to question. The problem of determining the structures or configurations of chemical molecules which react or combine with each other does not, from the evidence available from other fields of chemistry, depend upon the similarity of the reacting molecules or groupings. In fact, there is just as much, if not more, evidence for chemical reaction occurring with unlike molecules or groups or atoms. The analogy, therefore, while interesting as giving a mechanical picture for certain observations of specificity, cannot be used as a means of determining the structure

[4] E. Fischer, *Ber.* **27**, 2985 (1894); cf. E. Fischer and H. Thierfelder, *Ber.* **27**, 2036 (1894).

or configuration of the enzyme or active enzyme grouping. It is possible that the further study of the electronic structures of reacting molecules will throw light on these relations.

The question of adsorption as a possible explanation for enzyme actions was discussed at some length in the earlier chapters. It may suffice to state here that in the opinion of the writer adsorption represents only a step in the mechanism of enzyme actions and is not the dominating factor; that chemical considerations must be used to account for the various steps and combinations (including adsorption) involved, and that, whatever part adsorption may play is due mainly if not entirely to the chemical properties and reactions taking place with the adsorbing surface and adsorbed substance.

It has been found that various materials occurring in natural sources, sometimes being present together with the enzyme, possess the property of greatly increasing the enzyme action. For example, bile salts increase the action of pancreatic lipase. Such substances are generally known as co-enzymes in enzyme literature. It would appear that any material which increases the action of an enzyme preparation may be termed a co-enzyme. Co-enzymes have not been mentioned heretofore in this book. Substances which increase enzyme actions have been referred to a number of times. There is apparently no way of deciding where the actions of such substances might be said to end and those of co-enzymes begin. Strictly speaking, they should all be called by the latter name. Although this may fill a psychological need, it seems better not to use a new term for such an action unless it is shown that a new or different chemical phenomenon is involved. In looking about for analogous chemical reactions, it may be pointed out that in certain reactions of inorganic chemistry it was found that a mixture of two or more substances can increase the velocities of certain changes to a greater extent than any one of them can increase the velocity of these same changes separately. A well-known reaction is that involved in the action of the mixed earths in a Welsbach mantle, a mixture of 99% thoria and 1% ceria being found to be most efficient. A more recent striking example is that in which it was found possible to oxidize carbon monoxide to carbon dioxide at room temperatures with great rapidity by means of a mixture of various (four) oxides.[5] The number of these examples could be multiplied greatly. The term

[5] A. B. Lamb, W. C. Bray, and J. C. W. Frazer, *J. Ind. Eng. Chem.* **12**, 213 (1920).

"promoter" action in catalysis has come into use to indicate the influence of various substances in increasing the actions of catalysts. The general conclusion seems to be that mixtures of catalysts, or of a catalyst with substances otherwise inert, can be found for certain reactions which produce immeasurably greater increases in the velocities of the reactions than do the single catalysts.

It seems as if co-enzymes shold be treated similarly. The nature of the substance termed co-enzyme is known in some cases and may be very simple in character, such as the sodium chloride action with amylase. In other cases it may be quite complex and be chemically unidentified, as in certain yeast fermentations. In view of these relations and the similar reactions in other fields of chemistry, it seems advisable to drop the term co-enzyme from the literature and to attempt to study the problems considered heretofore under that heading from a chemical standpoint.

The study of enzyme actions up to the present time has dealt mainly with problems having as their aim the determination of the chemical structure of enzymes and the careful investigation of the conditions governing the increases in velocities of the chemical reactions influenced by enzymes. The methods, results, and theories developed in other branches of chemistry have been used wherever possible. It may be asked now whether the study of enzymes and enzyme actions has aided in the general development of chemical knowledge and theory aside from the specific problems being studied. It may be said that a beginning has been made in this way. Considering the short space of time in which it has been possible, because of the experimental difficulties involved, to carry on accurate enzyme studies, the promise for future usefulness is great.

The industrial uses of enzymes and some analytical work in which enzymes are used as reagents were referred to in Chapter VIII. In addition, enzyme studies have aided in clarifying certain general relations of theoretical chemistry. Reference is made to the topic of catalytic reactions primarily, and to the mechanism of chemical reactions in general secondarily. Enzymes have always been considered to be catalysts. The experimental evidence with regard to the mechanism of chemical reactions whose velocities are increased by enzymes points unmistakably to the primary formation of compounds between enzyme and substrate, followed by the decomposition into enzyme and products. The possibility of showing

this, is due to the nature and properties of the various components involved in the actions. All the steps are not clear in some of the reactions studied, but the general truth of the mechanism outlined seems to be based upon satisfactory evidence. With inorganic, and very frequently with organic, reactions which are catalyzed by added substances, the evidence for such addition compound formation is not readily obtainable. However, as more careful work is being described in these fields, it is found that an increasing number of reactions show this addition compound formation. The proof is ordinarily more difficult than with enzyme actions because of the properties of the substances and mixtures. As a result of all this work it may be stated that the most probable theory for the mechanism of chemical reactions in which a catalyst takes part involves the formation of an intermediate addition compound with the catalyst. Since a catalytic reaction is defined or described as a chemical reaction in which the chemical composition of one of the products of the reaction is the same as that of one of the initial components, the next step in the chemical theory would include in such a mechanism of chemical reactions all reactions between two or more substances, whether or not the composition of one or more of the substances is unchanged as a result of the reaction. This is the addition theory of chemical reactions. As the material for enzyme actions is presented in this monograph, it would appear as if this theory was assumed initially and that enzyme actions were assigned a place in it. Logically, however, the matter is reversed, and enzyme actions can really be used as a line of evidence in favor of the addition theory. The method of presentation used here is simply a matter of convenience to show the nature of enzyme actions, their relation to chemical reactions in general, and the possibilities for further study.

It is possible that further advances of analogous nature will result from the careful study of enzyme actions. The recent work of Northrop which was described in Chapter VIII may be referred to in this connection, as developing some new views on enzyme actions which will undoubtedly be applicable in some form to other reactions. At the present time, reactions of organic chemistry are throwing considerable light on reactions of inorganic chemistry. For example, the mechanism involved in the apparently simple solution of a metal in an aqueous solution of an acid is being cleared

up by the study of the Barbier-Grignard reaction. This is not the place to enter into these questions, but the relations are interesting and promise much for the future.

A series of changes in which enzyme actions unquestionably play a part include those involving carbohydrate transformations in plants and animals. Some of these changes have been referred to in earlier chapters. Leaving aside the glucose synthesis in plants probably from carbon dioxide and water, and considering only the relations between hexoses and the polysaccharides, in plants the interconversions of celluloses, starches, intermediate polysaccharides and monosaccharides must be considered; in animals, mainly the relation between glycogen and the hexoses. Such substances as the glycoproteins need not come into consideration in the first instance. Enzyme actions are to be expected in the various materials in which these changes occur *in vivo*. Amylases have been found in a number of plants, but it is a striking fact that extended investigation has failed to show satisfactory evidence of such an enzyme in the banana,[6] although there the conversion of starch into simpler carbohydrates is remarkably rapid. It is possible that in these changes an oxidation action is involved and not alone an amylase action. These results are quoted as possibly indicating complicated enzyme and other actions where comparatively simple actions might have been expected. The study of the enzyme actions involved in the carbohydrate changes in plant and animal life offer interesting possibilities. Up to the present very little appears to have been done in correlating the enzyme actions with the chemical changes which occur. The recent work on the chemical structures of the various carbohydrates and the relations between them will undoubtedly aid in the more intensive studies of the corresponding enzyme actions.

In concluding this monograph, some of the direct applications of enzymes in living matter may be mentioned. The results given in Chapter IX and the method of working there described bear directly upon these problems. As far as can be told, the chemical properties of substances in living matter are identical with their properties as obtained in the laboratory. The characteristic of substances in living matter is change, and change in certain definite ways. Enzymes are derived from living matter. They induce

[6] K. G. Falk and G. McGuire, *J. Gen. Physiol.* **3**, 595 (1920-21).

changes in definite directions in substances of more or less complex character. It does not require a far stretch of the imagination to consider enzymes the essential feature of living matter—since living involves chemical changes in certain directions. Also since enzymes are produced in living matter, the actions appear to be self-perpetuating, in that outside directive agencies are not required to produce the special enzymes needed to bring about the chemical reactions necessary for the continuation of life processes. The interest in enzymes and enzyme actions from the point of view of life processes is therefore justified because of their importance as the directive influences in the chemical reactions of living matter.

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